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Studies into the Disparity in Extent of Human Herpesvirus 8 Infection in Saudi Arabian General Population, Chronlc Renal Failure Patients and Renal Allograft Recipients

Submitted in fulfilment of the conditions governing candidates for the degree of

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Abstract

Transplantation-associated Kaposi's sarcoma (KS), causatively associated with human herpes virus 8 (HHV-8), is particularly prevalent in Saudi Arabia, although the prevalence rate of HHV-8 infection in the general population there is comparable to that in other geographical regions. The serologic and genomic prevalences of HHV-8 in samples representing the general population (n=238), patients with end-stage renal disease (n=78), and renal allograft recipients (n=66) were investigated. To evaluate if oral shedding of HHV-8 might play a role in transplantation KS, the extent of HHV-8 shedding in the mouth compared to other anatomical compartments, and the presence of multiple HHV-8 infection were also studied. PCR protocols were applied to amplify 3 fragments of the viral genome (from open reading frames 26 and K1) from whole-mouth saliva, parotid saliva, buccal and palatal exfoliates, plasma, sub-sets of peripheral blood cells, and KS lesional tissue, and to quantify the salivary viral load. Demographic and clinical data were analysed to identify risk factors for HHV-8 infection. A higher HHV-8 seroprevalence was observed in patients with renal disease compared to the general population, but no significant difference in HHV-8 DNA detection rates in CD45+ cells was found. The oral cavity was identified as a major site of HHV-8 shedding in renal disease patients regardless of a previous history of KS. In patients with end-stage renal disease, HHV-8 DNA was more frequently detectable in oral samples than in blood. They and renal allograft recipients showed evidence of being multiply infected by HHV-8. These findings suggest that iatrogenic, salivary HHV-8 transmission between patients with renal disease prior to transplantation accounts for the relatively high prevalence of HHV-8 infection. Implementation of measures to minimise contamination of oral fluid between renal disease patients may play a role in controlling HHV-8 transmission and reduce the incidence of transplantation-associated KS.

Table of contents

Declaration

The findings reported in this thesis result entirely from my own work. Colleagues who helped in various aspects of the work are listed in the Acknowledgments. The work has not previously been submitted, in part or in full, for a degree or diploma of this or any other University or examination board.

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Finally, I would like to dedicate this thesis to my parents, husband and children for their constant prayers, patience and support.

List of Figures

List of Tables

Abbreviations

AIDS	Acquired immunodeficiency syndrome
Ang2	Angiopoietin-2
ART	Anti-retroviral therapy
Bad	Bad protein, promotes cell death by displacing Bax protein
Bak	Bak integral membrane protein, promotes cell death
BAL	Bronchoalveolar lavage
Bax	Bax protein, 21 kDa homologous partner of Bcl-2
BCBL	Body cavity based lymphoma
Bcl-2	B cell leukaemia / lymphoma-2 genes
bFGF	Basic fibroblast growth factor
bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
CC	Subfamily of chemokines which have 2 adjacent cysteine reidues
CCR	CC chemokine receptor
CD	Cluster of differentiation
cdk	Cyclin dependent kinase
CI	Confidence intervals
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CXC	Subfamily of chemokines which have 2 cysteine residues separated by single amino acid
d	Day
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
Ds	Double stranded
DX	Diagnosis
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra-acetate
EHV	*Equine herpesvirus*
ELISA	Enzyme linked immunosorbant assay
ESRD	End-stage renal disease
FLICE	Fas-associated death domain-like interleukin-1 beta-converting enzyme
FLIP	FLICE inhibitory protein
GC	Guanine-cytosine

GPCR	G-protein-coupled receptor
h	Hour
HBV	Hepatitis B virus
HCV	Hepatitis C virus
H-DNA	High GC content
HHV	Human herpesvirus
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
HHV-8	Human herpesvirus 8
HIV	Human immunodeficiency virus
HPV	Human papilloma viruse
HSV	Herpes simplex virus
HV	Herpes viruses
HVA	Ateles virus of spider monkeys
HVS	Herpesvirus saimiri
ICAM	Intercellular adhesion molecule
ICTV	International Committee on the Taxonomy of Viruses
IDU	Injecting drug user
IFA	Immunofluorescence assay
IFN	Interferon
IL-6	Interleukin 6
IL-8	Interleukin 8
IRES	Internal ribosome entry site
IR	Internal repeat
IRF	Interferon regulatory factor
kb	Kilobase
kbps	Kilobase pairs
kDa	Kilodaltons
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
LANA	Latency-associated nuclear antigen
L-DNA	Low GC content
LTR	Left terminal repeat
LUR	Long unique region
MCD	Multicentric Castleman's disease
MCMV	Murine herpesvirus 1
MCP	Major capsid protein
MDa	Megadalton

µg	Microgram
mg	Milligram
MHV-1	Mouse cytomegalovirus 1
MHV-68	*Murine herpesvirus strain 68*
min	Minutes
MIP	Macrophage inhibitory protein
µl	Microlitre
ml	Millilitre
mM	Millimolar
MPC	Magnetic particle concentrator
mRNA	Messenger RNA
NF	Nuclear factor
NFAT	Nuclear factor of activated T-cells
nm	Nanometre
OD	Optical density
OR	Odds ratio
ORF	Opening reading frame
p53	Protein 53, tumour suppressor gene
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
PKR	Protein Kinase
pRb	Retinoblastoma tumour suppresser protein
PRV	*Pseudorabies virus*
PTLD	Post-transplant lymphoproliferative disorder
RDA	Representational difference analysis
RNA	Ribonucleic acid
rpm	Revolutions per minute
RRV	*Rhesus rhadinovirus*
Rta	Replication and transcription activator
RTR	Right terminal repeat
RV	Rhadinovirus
s	Seconds
SD	Standard deviation
SHV-2	Simian herpesvirus 2
SSCP	Single-strand conformational polymorphism
TAE	TRIS-acetate-EDTA

Taq	*Thermus aquaticus*
Tat	Transactivator protein
TBE	TRIS-borate-EDTA
TGF-beta	Transforming growth factor-beta
T_m	Melting temperature
TNF-alpha	Tumour necrosis factor alpha
TPA	12-*O*-tetradecanoylphorbol 13 acetate
TR	Terminal repeat
U	Unique sequence
UL	Long unique sequence
UPGMA	Unweighted pair group method with arithmetic means
US	Short unique sequence
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VR	Variable region
VZV	Varicella-zoster virus
wk	Week
yr	Year

Chapter 1

Introductory Considerations

1.1 Overview of herpesviruses

1.1.1 General features

Herpesviruses are a family of DNA viruses found commonly in humans and animals. They are widely disseminated in nature and most animal species carry at least one herpesvirus and frequently several, diverse herpesviruses. The number of herpesviruses in nature is likely to exceed the 130 herpesviruses identified to date. Herpesviruses share the following four biological properties:

(i) Synthesis of viral DNA and assembly of their capsid occur in the nucleus of infected cells.

(ii) They encode their own enzymes involved in nucleic acid metabolism and synthesis and protein processing - although the array of enzymes may vary from one herpesvirus to another.

(iii) Production of infectious viral particles is invariably accompanied by destruction of the infected cell.

(iv) Latent infection that persists for the life of their natural hosts. The latent viral genomes usually take the form of circular episomes, with only a small subset of viral genes being expressed. Latent genomes retain the capacity to replicate and cause disease upon reactivation. The site of latency differs from one herpesvirus to another.

1.1.2 Structure of Herpesviruses

1.1.2.1 The virion

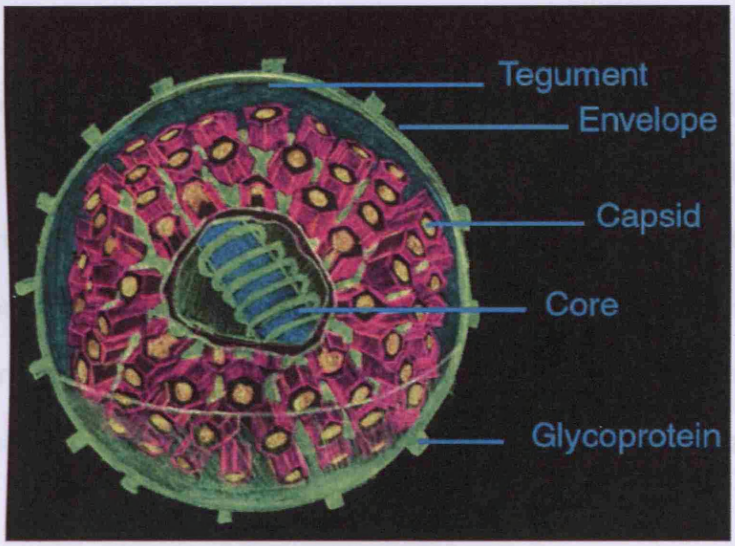
Herpesviruses have a common virion architecture (Figure 1.1) consisting of:

- **Core,** containing linear double-stranded DNA.
- **Capsid,** surrounding the core and containing 162 capsomeres. It is icosahedral in shape and approximately 100 to 110nm in diameter.
- **Tegument,** an amorphous-appearing material between the capsid and the envelope.

- **Envelope,** forming the outer layer of the virion and containing viral glycoprotein spikes on its surface.

Herpesvirions vary in size from 120 to nearly 300 nm. The variation is in part due to variability in the thickness of tegument. Another major source of variability is the state of the envelope. Intact envelopes are impermeable and generally retain the quasi-spherical shape of the virion during preparation for electron microscopy. Damaged envelopes have a diameter much larger than that of intact virions (reviewed by Roizman & Pallett 2001).

Figure 1.1 Schematic structure of a herpesvirus



[Source: (Solomon, 2007)]

1.1.2.2 Genomic organisation

Herpesvirus genomes vary in length from 120 to 250 kilobase pairs (kbps) and contain 60 to 120 genes. Herpesvirions contain, in addition to viral DNA, 25-35 virus-encoded proteins and host-specific phospholipids derived from the nuclear membrane.

The length of the genome is characteristic for each genus. Herpesviruses can be distinguished from each other not only by the size and base composition of their DNA, but also by the structural arrangement of their unique and repeated sequences. Variations in the number of internal and terminal repeat regions result in minor variations in the size of individual isolates.

Herpesvirus genes may be characterized as either essential or dispensable for growth in cell culture. Essential genes regulate transcription and are required to construct the virion. Dispensable genes for the most part function to enhance the cellular environment for virus production, to defend the virus from attack by host immune system and to promote cell-to-cell spread. All herpesvirus genomes contain lengthy terminal repeats, both direct and inverted (reviewed by Roizman & Pallett 2001).

Herpesviruses can be divided into six structurally distinct groups on the basis of the presence and location of repeated sequences longer than 100 base pairs (bps) (Figure 1.2):

(i) Group A viruses possess a large sequence in one terminus which is directly repeated at the other terminus. They include human herpesviruses 6 and 7 (HHV-6 and HHV-7).

(ii) Group B viruses possess a terminal sequence that is directly repeated numerous times at both termini. Typically an area of low GC content (L-DNA)

is flanked by repetitive regions of high GC content (H-DNA). Examples include human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), and the primate herpesvirus saimiri (HVS), also known as simian herpesvirus 2 (SHV-2).

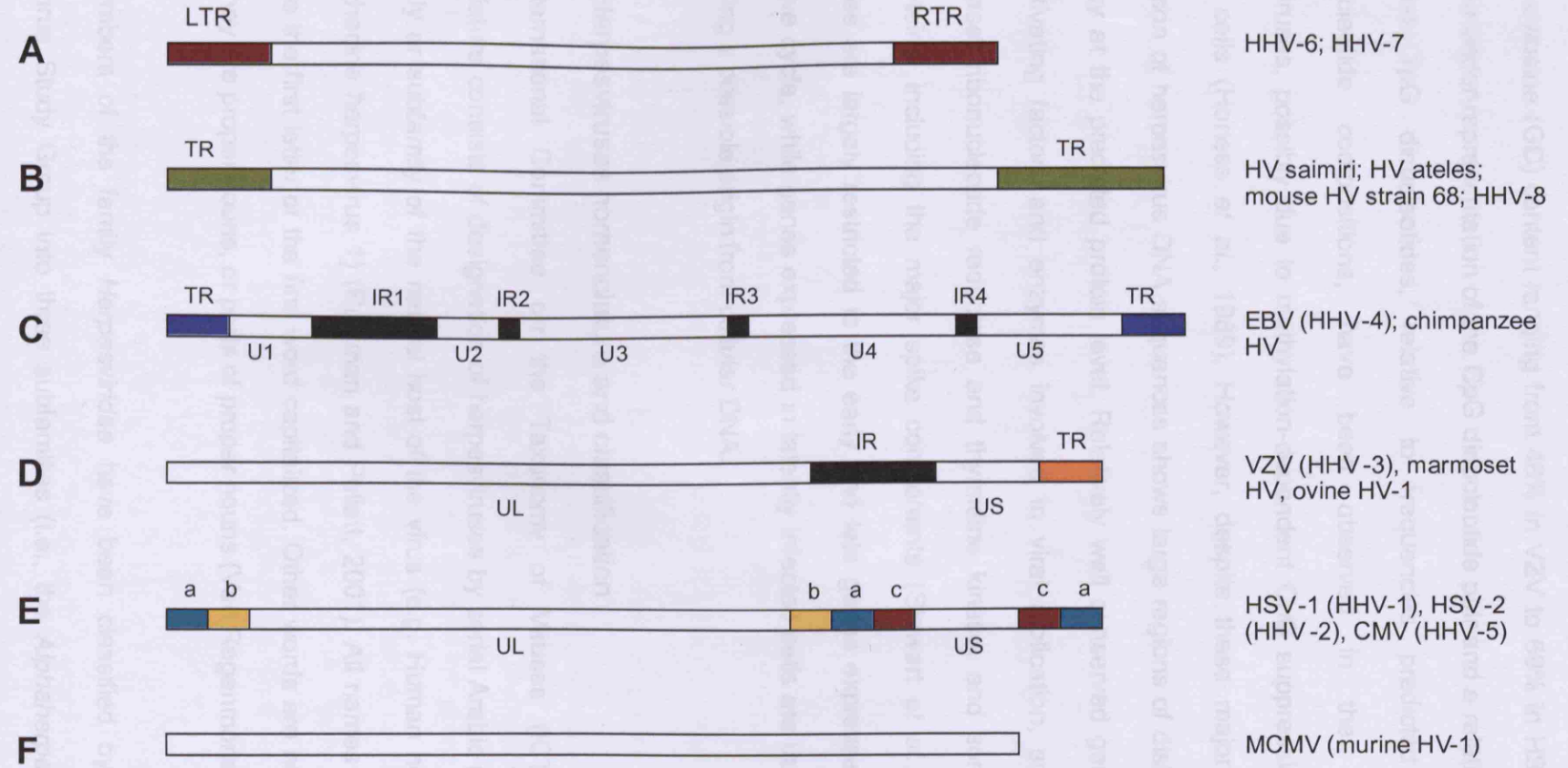
(iii) Group C viruses, for which both terminal and internal repeat sequences are present throughout the viral genome, and which further subdivide it into well-defined unique sequences. These include Epstein-Barr virus (EBV) and chimpanzee herpesvirus (pongine herpesvirus 1).

(iv) Group D viruses have a terminal region which is repeated in an inverted orientation internally. Examples include varicella-zoster virus (VZV) and a large number of viruses isolated from mammals and birds.

(v) Group E viruses have a more complex genomic structure, in which sequences at both termini are repeated in inverse orientation and juxtaposed internally. They include herpes simplex viruses types I (HSV-I) and 2 (HSV-2) and human cytomegalovirus (CMV).

(vi) Group F viruses have no repeat regions in the genome, and are exemplified by murine herpesvirus 1.

Figure 1.2 Architecture of herpesvirus genomes



Abbreviations: LTR, left terminal repeat; RTR, right terminal repeat; TR, terminal repeat; IR, internal repeat; U, unique sequence; UL; long unique sequence; US, short unique sequence; repeat sequences, a,b,c.

The herpesviruses also vary significantly in terms of their base composition, with the guanine-cytosine (GC) content ranging from 46% in VZV to 69% in HSV (Roizman, 1996). An under-representation of the CpG dinucleotide pair and a relative excess of CpA and TpG dinucleotides, relative to frequencies predicted from their mononucleotide compositions, have been observed in the lymphotropic herpesviruses, possibly due to methylation-dependent CpG suppression in rapidly dividing cells (Honess *et al.*, 1989). However, despite these major differences, comparison of herpesvirus DNA sequences shows large regions of distant co-linear homology at the predicted protein level. Relatively well conserved genes code for *trans*-activating factors and enzymes involved in viral replication, such as DNA polymerase, ribonucleotide reductase and thymidine kinase, and some structural glycoproteins, including the major spike components (Stewart *et al.*, 1996). The similarities are largely restricted to the early and late genes expressed during the productive cycle, while genes expressed in latently infected cells are usually unique, suggesting a possible origin from cellular DNA.

1.1.3 Herpesviruses nomenclature and classification

The International Committee on the Taxonomy of Viruses (ICTV)-endorsed nomenclature consists of designation of herpesviruses by serial Arabic numbers and the family or subfamily of the natural host of the virus (e.g. Human herpesvirus 7, Cercopithecine herpesvirus 1) (Roizman and Pellett, 2001). All names are in italics and have the first letter of the first word capitalized. Other words are not capitalized unless they are proper nouns, or parts of proper nouns (Van Regenmortel, 1999).

The members of the family *Herpesviridae* have been classified by the ICTV's Herpesvirus Study Group into three subfamilies (i.e., the *Alphaherpesvirinae,* the *Betahepesvirinae*, and the *Gammaherpesvirinae*) based largely on host range and tropism, as well as *in vitro* growth rate and characteristics of viral latency (Roizman *et*

al., 1981; Chee and Barrell, 1990; Murphy *et al.*, 1995) (Table 1.1 & Fig 1.3). Further subdivision into genera is based on DNA sequence homology, similarities in genomic sequence arrangement and viral proteins demonstratable by immunological methods (Roizman *et al.*, 1981; Roizman, 1982) .

1.1.3.1 Alphaherpesviruses

The subfamily *Alphaherpesvirinae* includes the genera *Simplexvirus* (HSV-1 & HSV-2), *Varicellovirus* (VZV), *Equine herpesvirus 1* (EHV-1) and *Pseudorabies virus* (PRV). They have a relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and are able to establish latent infection primarily, but not exclusively, in sensory ganglia.

1.1.3.2 Betaherpesviruses

The subfamily *Betaherpesvirinae* includes the genera *Cytomegalovirus* (CMV), *Muromegalovirus* (MHV-1), and *Roseolovirus* (HHV-6 & HHV-7). Betaherpesviruses replicate in a variety of cell types *in vivo*, including epithelial cells, while the host cell range is more restricted *in vitro*. Infection progresses slowly and is accompanied by cell enlargement (cytomegaly) and by the appearance of characteristic nuclear eosinophilic inclusion bodies, formed by the accumulation of defective particles containing enveloped viral proteins without DNA (also referred to as assembled capsids). Latent infection has been confirmed in many tissues, although lymphoreticular cells, secretory glands and renal tissue are the more common sites.

1.1.3.3 Gammaherpesviruses

The subfamily *Gammaherpesvirinae* includes the genera *Lymphocryptovirus* and *Rhadinovirus*, with little nucleotide sequence homology or antigenic cross-reactivity existing between these two groups. Gammaherpesviruses are characterized by their

tropism for lymphoid cells and their capacity to induce cell proliferation *in vivo*, resulting in transient or chronic lymphoproliferative disorders, and *in vitro*, where many can immortalise the infected cells. They have a narrow natural host range which is restricted to the family or order to which the natural host belongs. Most Gammaherpesviruses replicate inefficiently in haematopoietic cells, but some have efficient productive cycles in epithelial cells and fibroblasts. Latent virus is usually detected in lymphoid organs (Campbell *et al.*, 2005).

The lymphocryptoviruses (or gamma-1 herpesviruses) include EBV and related viruses of Old World primates such as chimpanzees (*Herpesvirus pan*), orangutans (*Herpesvirus orangutan*) and gorillas (*Herpesvirus gorilla*). These viruses share a tropism for B lymphocytes (Chee and Barrell, 1990), and a genomic architecture characteristic of group B or C, and a similar gene organisation. Furthermore, several of their structural and nonstructural proteins are antigenically related resulting in the elicitation of cross-reactive antibodies (Gerber and Birch, 1967; Chu *et al.*, 1971).

The rhadinoviruses (or gamma-2 herpesviruses) are classically the herpesviruses of primates. The extreme intragenomic GC heterogeneity, resulting in fragmentation by density centrifugation, led to the coining of the term *rhadinivirus* (meaning fragile, in Greek) (Roizman *et al.*, 1981). They can be divided into two distinct lineages, RV1 and RV2. Examples include the ateles virus of spider monkeys (HVA), the *Saimiri virus of squirrel monkeys* (HVS), *Rhesus rhadinovirus* (RRV), some viruses of horses e.g. *Equine herpesvirus 2* (EHV2) (Telford *et al.*, 1993) and mice, e.g. *murine herpesvirus strain 68* (MHV-68) (Sunil-Chandra *et al.*, 1994). The only human herpesvirus in this group is *human herpesvirus 8* (HHV-8) (Chang *et al.*, 1994). It has been classified in this genus due to its close similarity to HVS. HHV-8 is an RV-1 gamma-2 herpesvirus. There may be as yet undiscovered viruses of the RV-2 lineage capable of infecting humans (Lacoste *et al.*, 2001).

Table 1.1 Taxonomy of herpesviruses

	Alphaherpesvirinae:
Simplexvirus	Human herpesvirus 1, 2 (HSV-1, HSV-2)
Varicellovirus	Human herpesvirus 3 (VZV)
	Betaherpesvirinae:
Cytomegalovirus	Human herpesvirus 5 (CMV)
Muromegalovirus	Mouse cytomegalovirus 1 (MHV-1)
Roseolovirus	Human herpesvirus 6, 7 (HHV-6, HHV-7)
	Gammaherpesvirinae:
Lymphocryptovirus	Human herpesvirus 4 (EBV)
Rhadinovirus	Human herpesvirus 8 (HHV-8)

Figure 1.3 Classical neighbour-joining phylogenetic tree of near full length genome isolates demonstrating the relationship between different herpesvirsuses

[Source: Stebbing *et al.*, 2006]

Within these three subfamilies of herpesviruses, eight are known to infect humans. These are summarised in Table 1.2

Table 1.2 Human Herpesviruses

[Source: Roizman and Pellett, 2001]

1.2 Physical and genomic structure of human herpesvirus 8

1.2.1 The discovery of HHV-8

A viral aetiology for Kaposi's sarcoma (KS) was suspected long before the onset of the acquired immunodeficiency syndrome (AIDS) epidemic (Oettle, 1962). During the 1970s herpesvirus-like particles, thought to be CMV, were found by electron microscopy in tumour cells (Giraldo *et al.*, 1972). Subsequent studies identified, in lesional tissues, the DNA of CMV, HHV-6, human papilloma viruses (HPVs) and other viral or bacterial pathogens, all of which were put forward as candidate aetiological agents. However, only a minority of KS lesions was found to carry DNA of the implicated viruses (Huang *et al.*, 1992; Kempf *et al.*, 1995; Monini *et al.*, 1996c).

Although no aetiologic agent was identifiable, epidemiologic studies in the late 1980s suggested that a sexually transmitted agent was the cause of KS, this being transmitted independently of human immunodeficiency virus (HIV). The suspected agent was presumed to be more efficiently transmitted through homosexual than heterosexual contact, resulting in a much higher incidence rate among gay male AIDS patients than other AIDS risk groups. The agent was also likely to be poorly transmissible through blood products since KS prevalence rates among HIV-infected transfusion recipients and haemophiliacs were relatively low (Beral *et al.*, 1990; Beral, 1991; Beral *et al.*, 1992).

Given that there may be a separate KS agent, it was reasoned that the genomic difference between proposed infected (KS) and uninfected (skin) tissues from an individual patient with AIDS-KS should be that of the infectious agent. A subtractive hybridization technique, representational difference analysis (RDA), based on polymerase chain reaction (PCR) (Lisitsyn *et al.*, 1993) enrichment of unique DNA fragments was employed by Chang and colleagues (Chang *et al.*, 1994). With the use of genomic DNA from an AIDS-KS lesion that was compared to unaffected DNA

from the patient, two unique DNA fragments were isolated. These two DNA fragments (KS330Bam and KS631Bam), which showed high homology to known herpesvirus sequences, were used as Southern hybridization probes and for the generation of PCR primers. Both segments were homologous to two members of the gammaherpesvirus sub-family. KS330Bam was 51% homologous to opening reading frame (ORF) 26 of HVS and 39% homologous to ORF BDLF1 of EBV. KS631Bam was homologous to a tegument protein coded by ORF 75 of HVS and ORF BNRF1 of EBV. The unique sequences were found in 90% of KS lesions from AIDS patients. Subsequent studies showed that the vast majority of KS lesions from patients with AIDS and from other groups at risk of KS carried DNA of this novel virus (Ambroziak *et al.*, 1995; Schalling *et al.*, 1995; Boshoff *et al.*, 1995b; Chang and Moore, 1996). HHV-8 is therefore the most recently discovered human tumour virus and has been detected in virtually all cases of KS.

1.2.2 Morphology

The morphological characteristics of HHV-8 are typical of herpesviruses (Figure 1.1). These features were confirmed in electron micrographs of HHV-8-infected primary effusion lymphoma (PEL) cell lines (Arvanitakis *et al.*, 1996; Renne *et al.*, 1996b; Said *et al.*, 1996b), and biopsy samples of KS (Walter *et al.*, 1984; Orenstein *et al.*, 1997). HHV-8 appears as 100-150 nm particles, has a target-like ultrastructural appearance with an electrondense central core surrounded by a lipid envelope (Renne *et al.*, 1996b). Capsid substructures, such as ring-shaped capsomers of approximately 9 nm in diameter arranged in linear arrays, have also been observed (Arvanitakis *et al.*, 1996; Said *et al.*, 1996b).

The capsid composition of *Gammaherpesvirinae*, including HHV-8, has since been characterised by means of biochemical and imaging analyses. It has been confirmed that only a portion of the synthesised capsids undergoes viral DNA packaging during

the lytic replication phase. As with the alpha- and betaherpesviruses, this leads to the formation of at least three capsid species, A, B and C, with masses of approximately 200, 230, and 300 MDa, respectively. A capsids consist of an empty icosahedral shell; B capsids contain only scaffolding protein (ORF 17.5); and C capsids contain packaged DNA and no scaffolding protein. It has been hypothesised that the B capsids may mature to C capsids, which in turn may function as the precursors of infectious virus particles. The A capsids are probably the dead end products of B and C capsids (Nealon *et al.*, 2001).

Nealon and co-workers also confirmed that the hexameric and pentameric capsomers are composed of the major capsid protein (MCP) encoded by ORF 25. The heterotrimeric complexes, forming the capsid floor between the hexons and pentons, are each composed of one molecule encoded by ORF 62 and of two molecules encoded by ORF 26. These three proteins (encoded by ORF 25, 62 and 26) have a significant amino acid sequence homology to capsid proteins of both alpha- and betaherpesviruses. A fourth small, basic, and highly antigenic protein, encoded by ORF 65, was also identified, and in contrast does not show any significant sequence homology to its structural counterparts from the other subfamilies. A fifth protein, encoded by ORF 17.5, is only found in B capsids (Nealon *et al.*, 2001).

1.2.3 Genomic characteristics

Complete genome sequences of two HHV-8 isolates have now been determined, one from a PEL cell line (Russo *et al.*, 1996) and the other from a KS specimen (Neipel *et al.*, 1997), both revealing the characteristic co-linear genomic organization of rhadinoviruses. HHV-8 genome is 140.5 kb in length and is of low GC content (53.5%) (Figure 1.4). The linear double-stranded HHV-8 DNA comprises a segment called L-DNA or long unique region (LUR) that is surrounded by terminal repeat

regions (TRs) consisting of 801 bp direct repeat units with a high GC content (84.5%) called H-DNA. The L-DNA region of HHV-8 genome has at least 90 ORFs and five internal repeat regions (Russo *et al.*, 1996; Neipel *et al.*, 1997; Cohen *et al.*, 2005).

As with other gammaherpesviruses, essential ORFs that code for viral replication and assembly proteins are arranged in blocks and are referred to as the conserved genes. There are approximately 67 such genes conserved among members of herpesvirus subfamilies. In HHV-8, these include: ORF 25, coding for the major structural protein; ORF 9, for DNA polymerase; and ORF 17, for a proteinase and assembly protein (Moore *et al.*, 1996b). Non-conserved gene blocks in the HHV-8 genome lie between the conserved blocks and contain some ORFs that are found in other rhadinoviruses including homologues to mammalian proteins. Like other rhadinoviruses, HHV-8 has numerous open reading frames with striking homology to known cellular genes. All known rhadinoviruses have unspliced genes that seem to have been captured from the host cell during viral evolution. Typically, they code for proteins that interfere with the immune system, for enzymes involved in nucleotide metabolism, and for putative regulators of cell growth (Russo *et al.*, 1996; Neipel *et al.*, 1997; Neipel *et al.*, 1998). The HHV-8 genome has cellular homologues not found in other rhadinoviruses including a homologue to human interleukin 6 (IL-6), members of the CC chemokine family and interferon regulatory factors (IRFs). These gene products may contribute to the pathogenesis of HHV-8 and will be discussed in more detail in Section 1.3. Genes specific to HHV-8 lineage (McGeoch and Davison, 1999) are listed in Table 1.3.

Figure 1.4 The Human herpesvirus 8 (HHV-8) genome

Note: The genome consists of a long unique region (140.5 kb) encoding for over 80 open reading frames (ORFs), surrounded by terminal repeat regions (TRs) consisting of 801 base pair direct repeat units with a high G+C content. Three large regions (I, II and III) contain genes conserved among the *Rhadinoviruses*, whereas the regions between them contain unique genes. Many of these unique genes encode homologues for host cellular proteins (shown in red with dotted outlines; genes without known homologues are shown in yellow with dotted outlines). Genes that are potentially important in the pathogenesis of KS are labelled.

Abbreviations: CCP, complement control protein; v-cyc, viral D-type cyclin; vFLIP, viral FLICE inhibitory protein; vGPCR, viral G-protein-coupled receptor; vIL-6, viral interleukin 6; vIRF, viral interferon regulatory factor; kb, kilobase; LANA, latency-associated nuclear antigen; vMIP (vCCL), viral macrophage inflammatory protein

[Source: Expert Reviews in Molecular Medicine, 2001 Cambridge University Press]

Table 1.3 Genes specific to the HHV-8 lineage

[Source: McGeoch and Davison, 1999]

1.3 HHV-8 gene expression and encoded proteins of relevance

As with the other herpesviruses, HHV-8 exhibits two modes of replication: lytic, in which viral DNA is replicated by the virus-encoded polymerase and encapsidated into infectious virions; and latent, in which viral episomes replicate in tandem with host cell DNA using host cell replication machinery. Both modes of replication are characterized by virtually distinct gene expression programs. HHV-8 establishes latency in the host and is under stringent latent replication control in PEL cell lines (Russo *et al.*, 1996; Gao *et al.*, 1996a; Sarid *et al.*, 1998). While a minority of cells in culture may express lytic cycle proteins at any given time, chemical induction with tetradecanyl phorbol acetate (TPA) is needed for the full expression of lytic gene products in PEL cells *in vitro*. Three broad categories (classes) of gene expression have been described (Sarid *et al.*, 1998), based on their expression outcomes after TPA induction (although this may not reflect the true *in vivo* state): class I (which are unaffected by TPA induction and presumably represent latent viral transcripts); class II (which are expressed without TPA but are induced to higher transcription levels by TPA treatment and typically represent the virus encoded cytokines, signal transduction genes and small polyadenylated RNAs [T0.7 & T1.1]); and class III (which are primarily structural and replication genes that are transcribed only following TPA treatment and are presumably responsible for lytic virion production).

1.3.1 Latent infection

HHV-8 becomes latent in the majority of cells it infects. During this stage of the virus life cycle, the viral genome resides in the nucleus as closed circular episomal DNA expressing a small subset of viral genes. Potentially, this minimises the number of viral epitopes that are presented by infected cells to cytotoxic T lymphocytes (CTLs) (Renne *et al.*, 1996a). It seems likely that the genes expressed during latency play a major role in the pathogenesis of HHV-8. The major latent loci are as summarized below.

1.3.1.1 ORF 71 (K13)

ORF 71 produces vFLIP, a homologue to cellular FLICE (FADD [Fas-associated death domain]-like interleukin-1 beta-converting enzyme) inhibitory proteins (FLIPs). vFLIPs found in other herpesviruses are known to inhibit fas-mediated apoptosis by preventing the recruitment of caspase-8 (FLICE) to the death-induced signalling complex (Thome *et al.*, 1997). vFLIP in HHV-8 has a similar function and can prevent apoptosis in infected cells (Djerbi *et al.*, 1999; Sturzl *et al.*, 1999). ORF 71 utilises internal ribosome entry site (IRES)–mediated initiation to produce a functional protein and that ensures the protection of infected cells from CTL-induced cell killing throughout the cell cycle (Bieleski and Talbot, 2001). vFLIP also has the ability to transform Rat-1 and Balb/3T3 fibroblast cells by activation of the nuclear factor kappa B (NF-kappaB) pathway. NF-kappaB is a transcriptional regulatory factor that in turn up-regulates the expression of a wide variety of inflammatory cytokines and adhesion molecules, playing a pivotal role in promoting inflammation and cell proliferation (Pati *et al.*, 2001; Caselli *et al.*, 2006; Sadagopan *et al.*, 2007). This suggests that vFLIP has activity beyond its role as an inhibitor of death receptor signalling, and may play a causative role in the pathogenesis of HHV-8-associated malignancies (Sun *et al.*, 2003; Matta and Chaudhary, 2004; Chugh *et al.*, 2005; Matta *et al.*, 2006; Wang *et al.*, 2006; Sun *et al.*, 2006).

1.3.1.2 ORF 72

ORF 72 encodes viral cyclin (v-cyclin), which is the viral homolog to cellular cyclin and is most closely related to cyclin D. The HHV-8 v-cyclin has been shown to associate primarily with cdk6 and to a lesser extent with cdk4, both important cell cycle regulators (Li *et al.*, 1997; Radkov *et al.*, 2000). v-cyclin is able to interfere with the phase G1 of the cell cycle via the inactivation of retinoblastoma tumour suppresser protein (pRb) and the Cdk inhibitors p16 (Ink4a), p21Cip1 and p27Kip1 (Verschuren *et al.*, 2004), thereby enhancing the survival of HHV-8-infected cells

(Meyerson and Harlow, 1994; Swanton *et al.*, 1997; Ellis *et al.*, 1999).

HHV-8 v-cyclin is expressed in latently infected spindle cells and PEL cell lines, and in KS tissues (Cesarman *et al.*, 1996; Godden-Kent *et al.*, 1997; Davis *et al.*, 1997b; Reed *et al.*, 1998), suggesting that it may have a role in either the proliferation or the arrest of differentiation of these cells.

1.3.1.3 ORF 73

ORF 73 encodes the latency associated nuclear antigen-1 (LANA-1). LANA-1 is detected in the majority of KS lesions as well as in cell lines derived from PELs. LANA-1 plays a role in HHV-8 maintenance by tethering the viral episome through the terminal repeat to host chromosome, ensuring persistence in the daughter cells after successful rounds of cell division (Ballestas *et al.*, 1999; Cotter and Robertson, 1999; Radkov *et al.*, 2000; Stuber *et al.*, 2007). The terminal repeat DNA binding domain is localized to LANA-1 residues 996–1139 (Srinivasan *et al.*, 2004; Komatsu *et al.*, 2004), and interacts with SUV39H1 histone methyltransferase, a key component of heterochromatin formation (Sakakibara *et al.*, 2004; Stuber *et al.*, 2007). This interaction creates the characteristic pattern of intra-nuclear dots observed in immunofluoresence assays.

In normal uninfected cells, G1/S progression is negatively regulated by binding of pRb to E2F transcription factor. In infected cells, LANA-1 competes with E2F for binding of pRb, thus freeing E2F to activate the transcription of genes involved in cell cycle progression. LANA-1 also binds p53, a tumour suppressor gene involved in many types of human cancers (Vogelstein *et al.*, 2000), and blocks p53-mediated apoptosis, allowing HHV-8-infected cells to persist (Friborg, Jr. *et al.*, 1999; Barbera *et al.*, 2004). The amino terminus of LANA can interact with its carboxy-terminal domain. This interaction is potentially important for facilitating associations with other

cell cycle regulatory proteins (Kaul *et al.*, 2007). These novel associations render LANA a key factor in relation to the maintenance of latency and subsequent transformation of HHV-8 infected cells.

1.3.1.4 ORFs K10.5, K11.1 and K12

K10.5, K11.1 and K12 are considered as latent genes but can be induced by TPA during lytic replication of the virus, and may therefore be classified as class II transcripts (Sarid *et al.*, 1998; Jenner *et al.*, 2001). K10.5 expresses LANA-2 protein (also called vIRF-3). HHV-8 vIRF-3 interacts with cellular interferon regulatory factor 7 (IRF-7) and this interaction specifically suppresses IRF-7 DNA binding activity, thereby inhibiting the IFN alpha gene expression upon virus infection, suppressing both IFN-mediated and tumour suppressor-mediated host surveillance, which ensures a comprehensive avoidance of host anti-viral activity (Joo *et al.*, 2007). Interferons (IFNs) are a group of cytokines with wide ranging effects including defence against viral infection and inhibition of cell growth. Interferon regulatory factors (IRFs) regulate the transcription of IFNs thereby modifying the effects brought about by IFN activity (Reis *et al.*, 1992; Taniguchi *et al.*, 1995). HHV-8 vIRF-3 is expressed latently only in cells and cell lines (i.e. PEL, PEL cell lines and Multicentric Castleman's disease [MCD]) of haematopoietic origin, but not in KS tumours, possibly contributing to HHV-8 tumorigenesis in hematopoietic tissues. HHV-8 vIRF-3 is abundantly expressed in the nuclei of cultured HHV-8 infected B cells. Reporter assays showed that HHV-8 vIRF-3 inhibits p53 expression, like LANA-1, potentially contributing to the proliferation of infected B cells in MCD (Rivas *et al.*, 2001).

The ORF K11.1 gene encoding viral interferon regulatory factor 2 (vIRF-2) is latently expressed in PEL cell lines (Burysek *et al.*, 1999). While IRFs have the ability to bind nucleotides, vIRF-2 can bind oligonucleotides corresponding to NF-kappaB (Burysek *et al.*, 1999; Burysek and Pitha, 2001). vIRF-2 inhibits the effects of cellular IFN

through its interaction with a ds RNA activated protein kinase (PKR) (Burysek and Pitha, 2001). PKR is normally induced by IFN and mediates the anti-viral and anti-proliferative effects of IFN (Sen and Ransohoff, 1993). Down-regulating the antiviral response caused by PKR, vIRF-2 may be important for maintenance of viral latency (Burysek and Pitha, 2001).

The ORF K12 gene encodes Kaposin A, which has been shown to induce tumorigenic transformation and is expressed in the PEL cell lines, BCBL-1 and KS-1 (Sadler *et al.*, 1999; Muralidhar *et al.*, 2000; Kliche *et al.*, 2001).

1.3.2 Lytic infection

The majority of the HHV-8 genome remains silent during latent infection. The programme of gene expression during lytic replication was revealed by DNA array expression profiling of PEL cell lines without and with stimulation by TPA (Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001). The lytic genes mainly express class II and class III transcripts (Sarid *et al.*, 1998). The first group of genes expressed after the induction of lytic replication are typically regulators of gene expression, including the immediate early transactivators. Viral homologs of cellular genes involved in regulation or signal transduction are expressed after the viral regulatory genes but before those involved in DNA replication. The structural genes and those involved in virus expression and maturation are expressed later, generally after 24 h post-induction (Jenner *et al.*, 2001).

Class II transcripts major lytic genes that are transactivators and homologues of cellular genes are as follows:

1.3.2.1 ORFs 50, K8 and 57

The first groups of genes expressed after the induction of lytic replication are typically

regulators of gene expression including the immediate early transactivators ORF 50 (Rta or Lyta), ORF K8 (Zta or Kb-ZIP) and ORF 57 (post-transcriptional regulator of gene expression) (Jenner *et al.*, 2001). The ORF 50/Rta promoter region is heavily methylated in latently infected cells. Demethylation of the promoter by TPA induces lytic cycle replication (Chen *et al.*, 2001). It has been demonstrated that the ORF 50 protein is capable of stimulating HHV-8 lytic genes through transcription cascades resulting in replication of the virus *in vitro* similar to the Zta and Rta proteins of EBV (Renne *et al.*, 1996b).

1.3.2.2 ORFs K4, K4.1 and K6

ORFs K4, K4.1 and K6 (located at the left end of LUR) of HHV-8 encode three secreted chemokines (chemoattractant cytokines). Viral macrophage inflammatory proteins (vMIP-I, vMIP-II and vMIP-III) are encoded by ORFs K4, K4.1 and K6, respectively (Moore *et al.*, 1996a; Neipel *et al.*, 1997; Nicholas *et al.*, 1997). vMIP-I and vMIP-II share 50% similarity at the amino acid level (Moore *et al.*, 1996a) while vMIP-III is less closely related. The three chemokines have been shown to induce angiogenesis, suggesting their roles in the pathogenesis of KS (Luttichau *et al.*, 2007). vMIP-I binds selectively to the CCR8 chemokine receptor, expressed predominately on Th2 type T cells, and acts as a CCR8 agonist (Endres *et al.*, 1999; Dairaghi *et al.*, 1999), while vMIP-II acts as a specific agonist of CCR3 (Boshoff *et al.*, 1997) and vMIP-III of CCR4 (Stine *et al.*, 2000). All the three major chemokine receptors, CCR3, CCR4 and CCR8, are chemoattractant receptors for Th-2 lymphocytes and their recruitment, and inhibit the Th-1 immune response thus potentially protecting HHV-8-infected cells (Stine *et al.*, 2000).

1.3.2.3 ORF 16

ORF 16 of HHV-8 encodes a homologue of the cellular anti-apoptotic protein Bcl-2, with which it shares 16% sequence homology (Russo *et al.*, 1996; Sarid *et al.*, 1997;

Neipel *et al.*, 1997; Cheng *et al.*, 1997). These HHV-8 v-bcl-2 transcripts can be detected at low levels in KS lesions and PEL cell lines, and can be induced in PEL cell lines by TPA (Sarid *et al.*, 1997).

The Bcl-2 family of proteins act as either inhibitors or promoters of apoptosis, playing an important role in tissue homeostasis and the immune response (Chao and Korsmeyer, 1998). The prosurvival function of cellular Bcl-2 is a result of heterodimerization with proapoptotic family members such as Bak, Bax, and Bad. However, the mechanism by which v-bcl-2 protects against apoptosis was initially unclear. Early cellular assays indicated that v-bcl-2 does not homodimerize nor heterodimerize with proapoptotic Bcl-2 family members such as Bak and Bax (Cheng *et al.*, 1997). However, more recently, it has been confirmed that v-bcl-2 has its own distinct selectivity for binding BH3 peptides from the proapoptotic proteins Bak, Bax, and Bad (Cheng *et al.*, 1997; Huang *et al.*, 2002).

1.3.2.4 ORF 74

ORF 74 of the HHV-8 genome encodes viral G-protein-coupled receptor (vGPCR) and is most related to the human interleukin 8 (IL-8) receptors. HHV-8 GPCR is a constitutively expressed chemokine-like receptor exhibiting affinity for a wide range of chemokines and does not require activation through an agonist (Arvanitakis *et al.*, 1997). vGPCR expressed in NIH3T3 cells has been shown to induce tumour formation when injected into nude mice and conditional transgenic expression of vGPCR by cells of endothelial origin trigger an angiogenic programme *in vivo,* leading to development of an angioproliferative disease that resembles KS, and is essential for its progression (Jensen *et al.*, 2005; Thirunarayanan *et al.*, 2007). Before the discovery of HHV-8, AIDS-associated KS was already known to express elevated levels of vascular endothelial growth factor (VEGF) that lead to angiogenesis, and it was found that vGPCR-transfected cells secrete elevated levels of VEGF (Bais *et al.*,

1998).

HHV-8-GPCR is capable of interacting with a much broader array of chemokines of both the CXC and CC families than most mammalian receptors, and can be modified in its signalling activity by them (Couty and Gershengorn, 2004). Studies demonstrated that factors such as growth-related oncogene-alpha and -gamma can activate HHV-8-GPCR above its constitutive signalling level, and are, therefore, considered full agonists of the receptor (Arvanitakis *et al.*, 1997; Rosenkilde and Schwartz, 2000). In contrast, factors such as the HHV-8-encoded CC chemokine vMIP-II and human stromal cell-derived factor-1alpha (SDF-1alpha) are inverse agonists of HHV-8-GPCR since they inhibit HHV-8-GPCR constitutive signalling, converting the receptor from an active to an inactive state (Geras-Raaka *et al.*, 1998).

HHV-8-GPCR utilizes a variety of signalling pathways to modulate the biology of its host. It activates the transcription factor NF-kappaB in primary endothelial cells and in a KS-derived endothelial cell line (Pati *et al.*, 2001) and nuclear factor of activated T cells (NFAT) (Pati *et al.*, 2003). Both NF-kappaB and NFAT activation induce secretion of several growth factors and proinflammatory and proangiogenic cytokines (IL-6 and IL-8), and increase the expression of adhesion molecules (e.g. VCAM-1, ICAM-1) (Bais *et al.*, 1998; Pati *et al.*, 2001; Schwarz and Murphy, 2001; Pati *et al.*, 2003). It has been suggested that strategies to block vGPCR may represent a novel approach for the treatment of KS (Jensen *et al.*, 2005; Mutlu *et al.*, 2007).

1.3.2.5 ORF K9

In addition to the latently expressed vIRFs discussed above, HHV-8 encodes other homologues of cellular IRFs lytically. The ORF K9-encoded vIRF-1 is homologous to cellular IRFs. It inhibits cellular responses to IFNs (Gao *et al.*, 1997; Zimring *et al.*, 1998), and can down-regulate the transcriptional activation induced by IFNs alpha,

beta , and gamma (Li *et al.*, 1998). This activity suppresses the host's IFN-mediated innate immune response to viruses. vIRF-1 also acts as an oncogenic protein by transforming rodent fibroblasts *in vitro* and causing tumour formation in nude mice (Gao *et al.*, 1997). It has been demonstrated that vIRF-1 can bind to p53 (Nakamura *et al.*, 2001). By suppressing phosphorylation and acetylation of p53, vIRF-1 inhibits its activity and prevents apoptosis in the infected cell (Vogelstein *et al.*, 2000; Nakamura *et al.*, 2001). More recently, vIRF-1 has been shown to inhibit the transforming growth factor-beta (TGF-beta) signaling (Seo *et al.*, 2005). TGF-beta is known to control proliferation, differentiation and other functions in most cell types.

1.3.2.6 ORF K2

ORF K2 of HHV-8 encodes vIL-6, that is approximately 24% homologous to human IL-6 (hIL-6) (Moore *et al.*, 1996c). vIL-6 is readily secreted by HHV-8-infected cells in PELs during latency, MCD and, to a lesser extent, in KS lesions (Moore *et al.*, 1996c; Parravicini *et al.*, 2000). Similar to hIL-6, vIL-6 activates signal transduction pathways through its interaction with the gp130 receptor, but unlike hIL-6, does not need to interact with the IL-6 co-receptor (IL-6R) to activate signalling. However, interaction with both gp130 and IL-6R may increase signalling efficiency (Wan *et al.*, 1999) and more recently, it has been confirmed that vIL-6 is capable of utilizing human alpha receptor (IL-6Ralpha) as part of a hexameric complex with enhanced signaling potency (Boulanger *et al.*, 2004). Both hIL-6 and vIL-6 are able to inhibit apoptosis and induce B-cell proliferation (Moore *et al.*, 1996a). Mice inoculated with vIL-6-transfected NIH3T3 cells develop more highly vascularised tumours than control mice and the tumours also express higher levels of VEGF (Aoki *et al.*, 1999). HHV-8 vIL-6 and vGPCR have recently been found to upregulate expression of angiopoietin-2 (Ang2), a secreted proangiogenic and lymhangiogenic molecule, in lymphatic endothelial cells. This expression is thought to contribute to the high level of Ang2 in individuals with KS; hence molecular mechanisms regulating its expression might

present a target for future anti-KS therapeutics (Vart *et al.*, 2007).

A number of genes involved in nucleic acid synthesis are coded for in the HHV-8 genome, in common with all herpesviruses (Roizman and Pellett, 2001). Rhadinoviruses, including HHV-8, encode a larger number of these proteins compared to other herpesviruses (Virgin *et al.*, 1997). Some of these proteins include DNA polymerase (ORF9), thymidine kinase (TK) (ORF 21), dihydrofolate reductase (DHFR) (ORF 2) and ribonucleotide reductase (ORF 60 and 61) (Russo *et al.*, 1996; Neipel *et al.*, 1997; Nicholas *et al.*, 1998; Goudsmit *et al.*, 2000). This group of genes is expressed as class III products during the late lytic phase. Their function may be to replace or enhance cellular DNA biosynthesis to aid in virion production.

1.3.3 Other HHV-8-encoded proteins

1.3.3.1 ORF 26

ORF 26 is a late gene which encodes the structural protein TRI-2 (Nealon *et al.*, 2001). It displays lytic expression between 24 h to 48 h post-induction, with peak expression at 36 h (Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001). TRI-2 joins with TRI-1 (ORF62) to form the heterotrimeric capsid triplexes (Nealon *et al.*, 2001). The triplexes reside between the hexomeric and pentomeric MCP (ORF 25) capsomers and make up the floor of the capsid. Two molecules of TRI-2 and one molecule of TRI-1 join to form each of the capsid's 320 triplexes (Nealon *et al.*, 2001). TRI-2 is present in 640 copies per capsid and is the second most abundant and third largest structural component of the capsid. TRI-2 is 21% identical and 41% similar to the HSV-1 triplex component VP23 (Nealon *et al.*, 2001).

1.3.3.2 ORF K1

ORF K1 is the first gene at the left end of the HHV-8 genome. It codes for a 46-kDa highly glycosolated transmembrane protein with similarities to the immunoglobulin

receptor family (Neipel *et al.*, 1997; Lagunoff and Ganem, 1997; Lee *et al.*, 1998a; Stebbing *et al.*, 2006). It is expressed in the early lytic phase after induction by TPA in PEL cell lines and is thought to have a transforming potential (Lee *et al.*, 1998b). Expression of K1 induces focus formation and morphological changes in Rat-1 fibroblasts, and can immortalize common marmoset lymphocytes to IL-2-independent proliferation to induce lymphomas *in vivo*. While it has no homologues in other herpesviruses, there is an equivalent position in the HVS genome from which transforming proteins are encoded (Lee *et al.*, 1998b).

The K1 protein has been shown to initiate signalling pathways in B cells which result in the tyrosine phosphorylation of cellular proteins, mobilisation of intracellular calcium, and the activation of transcription factors such as NFAT (Lee *et al.*, 1998a; Lagunoff *et al.*, 1999; Damania *et al.*, 2000). It has also been shown to downregulate B-cell antigen receptor complex expression in the plasma membrane (Lee *et al.*, 2000). The amino terminus of the protein interacts with μ chains of B-cell receptor complexes to retain them in the endoplasmic reticulum, suggesting a role in the survival of HHV-8-infected cells. An additional role for the protein in mediating paracrine activation of cells in which it is expressed has been proposed (Samaniego *et al.*, 2001). They demonstrated that cells expressing the protein showed increased NF-B-dependent promoter activity. NF-B is responsible for activating a number of inflammatory responses. The K1 protein may therefore activate uninfected endothelial cells in a paracrine manner through the activation of NF-B-dependent promoters and secretion of inflammatory cytokines.

The K1 is a 289-amino acid hypervariable protein. K1 of most strains is conserved in the N-terminal region (amino acids 1-19, predicted to be the signal peptide sequence) and the C-terminal region (amino acids 227-276, predicted to be a hydrophobic transmembrane domain and a small intracellular domain), but is highly variable in the

central region (amino acids 20-226, a predicted extracellular domain). Of the central region, the domain between amino acids 51-92 (variable region 1 or VR1) and between amino acids 191-231 (variable region 2 or VR2) are the most heterogenous (Figure 1.5). Certain segments within and connecting these two blocks are conserved, presumably to maintain the tertiary structure of the protein (Meng *et al.*, 1999).

Figure 1.5 Schematic illustration of the primary structure of the HHV-8 K1 protein

The signal peptide (SP) and transmembrane (TM) regions are indicated by stippling, and the two hypervariable domains VR1 and VR2 are indicated by crosshatching. Within the cytoplasmic (Cyt.) domain, the immunoreceptor tyrosine-based activation motif (ITAM) is indicated by solid black. [Source: Hughes and Hughes, 2007]

1.4 Genomic variation among HHV-8 strains

Studies comparing HHV-8 sequences from both KS biopsies and PEL cell lines confirm that the genome is highly conserved, with only 0.1% nucleotide variation between sequences (Russo *et al.*, 1996; Moore *et al.*, 1996b; Neipel *et al.*, 1997; Nicholas *et al.*, 1997). Initial studies conducted on the genetic variability and polymorphism of HHV-8 focused on two small gene fragments of ORF 26 and ORF 75 from KS specimens. However, ORF 26 was soon found to be one of the more highly conserved genes with only 2% divergence over a 500-bp region and very few substitutions that result in a change at the amino acid level (Moore and Chang, 1995; Huang *et al.*, 1995; Boshoff *et al.*, 1995b; Marchioli *et al.*, 1996; Moore *et al.*, 1996b; Zong *et al.*, 1997). Limited sequence variation has also been found within two regions of ORF 75 (Zong *et al.*, 1997). In a combined analysis of several genomic regions, including ORFs 26 and 75, Zong *et al.* (1997) found up to 1.5% only overall nucleotide variation between isolates, and grouped these into three different main variants, provisionally termed A, B and C (Zong *et al.*, 1997). More recently, very low rates of sporadic variation were found within an extended ORF 26 region (965-bp) sequence at nucleotide positions other than the 26 characteristic polymorphorisms used to define the different genotypes (A/C, J, K/M, D/E, B, Q, R and N) (Zong *et al.*, 2007b).

ORF K1, by contrast, displays up to 16% nucleotide and 32% amino acid variability, with sequences clustering into four main genotypes, I to IV, based on phylogenetic analysis and distinctive deletions (Meng *et al.*, 1999). Zong *et al.* (1999) also identified four main genotypes/subtypes, but assigned them A, B, C and D, and further divided these into subgroups based on amino acid differences of at least 5%. A more recent comprehensive study of 139 samples, from which at least VR1 sequence data were available, has yielded 24 defined ORF K1 subtypes and confirmed that the subtypes correlate with ethnicity and geography (Zong *et al.*,

2002). This extreme polymorphism of ORF K1 has been exploited to investigate the genomic diversity of HHV-8 (Stebbing *et al.*, 2006; Hayward and Zong, 2007). Subtypes A and C are the most related, while subtype B is the most divergent (Hayward, 1999; Hughes and Hughes, 2007) (Figure 1.6). The B subtype is found predominantly in sub-Saharan Africa or in persons of African heritage, and has only 3 subgroups (B1, B2 and B3). Less frequently, African (Zong *et al.*, 2002; Treurnicht *et al.*, 2002; Hayward and Zong, 2007) and Brazilian samples (Nascimento *et al.*, 2005) carry A5 subtype ORF K1 sequences. The A and C subtypes have a wide distribution throughout Europe, USA, Asia and the Middle East. The C2 variants predominate in both Saudi Arabian renal transplant-associated KS (Hayward, 1999; Zong *et al.*, 1999) and classic KS from the USA and Scandinavia (Zong *et al.*, 2002). The C5 variant, which is thought to be a hybrid between A and C genotypes (Hayward, 1999; Zong *et al.*, 1999), has been identified in Saudi Arabian renal transplant-associated KS (Hayward, 1999; Zong *et al.*, 1999) and in Morrocan classical, post-transplant and AIDS-associated KS (Duprez *et al.*, 2006). The D subtype appears to be rare and isolated to the Pacific Island region (Poole *et al.*, 1999). Sequences recovered from a Brazilian Amerindian population showed a 25-30% variation in nucleotide sequence from types A to D. These have been assigned to a new variant termed 'E' (Biggar *et al.*, 2000). Subtype E is also hyperendemic in Ecuador (Whitby *et al.*, 2004) and Amerindians in French Guiana (Kazanji *et al.*, 2005). Two further subtypes, designated F and G, have recently been identified (Kajumbula *et al.*, 2006; Hayward and Zong, 2007). AIDS KS patients in the US are normally infected with subtypes A1, A4 and C3. The predominance of these few subtypes suggests a combination of multiple, but limited reactivation from endogenously infected descendants of immigrants from endemic parts of the world together with localized horizontal spread (Zong *et al.*, 1999; Zong *et al.*, 2002). An association of HHV-8 subtype A with homo/bisexual males has been reported in Brazil (Nascimento *et al.*, 2005).

Figure 1.6 Predicted domain structure and key features of ORF-K1

Hatched bars denote signal peptide and transmembrane (TM) domains. Predicted N-glycosylation sites (NXS/NXT) (solid triangles), the 12 conserved Cys residues (solid circles), locations of highly variable VR1 and VR2 domains and summary listing of major subtype amino acid difference values both within and outside of the VR blocks are indicated. [Source: Zong *et al.*, 1999].

In addition, a novel subtype has been identified only in South Africa, based on the analysis of the ORF 75 gene, and classified as subtype N (Alagiozoglou *et al.*, 2000). At least two major variants of HHV-8 can be distinguished using the K15 gene between the ORF75 and the terminal repeat, situated at the right-end of the genome. The two variants, named P (predominant or prototype) and M (minor) alleles, show only 33% amino acid sequence identity (Poole *et al.*, 1999; Hayward and Zong, 2007). The P allele is the more frequent among HHV-8 genomes characterized, and has been found in association with five K1 subtypes (A - E) (Poole *et al.*, 1999). The rarer M allele has thus far been found in association with the A, B and C subtypes (Poole *et al.*, 1999). The K15-M and K15-P alleles appear to share similar functions in spite of their extensive sequence difference, both inducing the activation of the Erk2 and JNK1 kinases, the NF-kappaB transcription factor and the expression of similar range of cellular inflammatory genes (Wang *et al.*, 2007).

Loci across the genome have been investigated to determine whether the clustering patterns as suggested by ORF K1 sequencing can be confirmed. Data from three major loci, ORF 26, T0.7/K12 and ORF 75, confirm that a linkage exists across the genome with ORF K1 subtype patterns. In most instances, the four major ORF K1 subtypes (A, B, C and D) can also be discriminated at these loci (Poole *et al.*, 1999). However, although there is a general trend for linkages at different loci across the HHV-8 genome, this is not always the case (Zong *et al.*, 2007a).

The wide geographical and ethnic distribution of ORF K1 subtypes suggests that the major subgroups originated in paleolithic times through isolation and founder effects, associated with the expansionary migrations of modern humans out of east Africa first into sub-Saharan Africa starting 100,000 years ago (B subgroup), then into South Asia, Australia and the Pacific Rim beginning 60,000 years ago (D/E subgroup), with very little subsequent remixing, and, finally, as two major branches into Europe and

North Asia (both via the Middle East) between 10,000 and 35,000 years ago (A and C subgroups) (Zong *et al.*, 1999; Stebbing *et al.*, 2006; Hayward and Zong, 2007). Within the A and C branches, individual variant clades (A1 to A10 and C1 to C7) probably arose between 10,000 and 12,000 years ago as human populations expanded out from a limited number of Eurasian Ice Age refuges (Hayward and Zong, 2007).

1.5 Tropism of HHV-8

1.5.1 Detection of HHV-8 in KS, PEL and MCD affected tissue

HHV-8 DNA has been found in biopsy samples of all forms of KS irrespective of geographic origin, age, or gender of the patient (Chang *et al.*, 1996; Kasolo *et al.*, 1997; Penn, 1997; Metaxa-Mariatou *et al.*, 2004; Mwakigonja *et al.*, 2007; Pak *et al.*, 2007). The HHV-8 infection load appears to be higher in oral than in AIDS-related cutaneous KS tissue (Pak *et al.*, 2007).

In the KS tissue, HHV-8 DNA is present in a latent form in the vascular endothelial and spindle cells (Boshoff *et al.*, 1995a; Decker *et al.*, 1996; Sturzl *et al.*, 1997; Staskus *et al.*, 1997; Schulz, 1998; Dupin *et al.*, 1999), in addition to lytic virus present in a low number of infiltrating inflammatory monocytes (Blasig *et al.*, 1997). Experiments using PCR *in-situ* hybridization reveal that HHV-8 can infect the atypical endothelial cells lining the vascular spaces in KS lesions and endothelial tumour (spindle) cells of fully developed, nodular KS lesions, but is not generally present in normal endothelial cells (Boshoff *et al.*, 1995a; Li *et al.*, 1996). This has been confirmed by microdissection studies (Boshoff *et al.*, 1995a). *In-situ* hybridization (Staskus *et al.*, 1997) and immunohistochemical studies localising with LANA (Rainbow *et al.*, 1997) have confirmed HHV-8 gene expression in KS spindle cells within tumours.

HHV-8 DNA has also been detected in PELs (Cesarman *et al.*, 1995b; Dupin *et al.*, 1999). PEL cell lines such as BCBL-1 and BC-3 carry HHV-8 in a latent form, and lytic replication can be induced by TPA (Renne *et al.*, 1996a; Schulz, 1998; Dupin *et al.*, 1999). It appears that the cellular tropism of HHV-8 varies with the lesion involved. Staskus *et al.* (1999) used IL-6 expression as a marker of HHV-8 involvement in KS, PEL and MCD. They demonstrated that although PEL parallels KS in the pattern of latent and lytic cycle viral gene expression, the predominant infected cell type in PEL is the B cell, compared to spindle and endothelial cells in KS. MCD differed from KS not only in the infected cell type (B-cell and T-cell lineage) but also in the pattern of viral gene expression, with only a few cells in the lesion infected and all of these cells expressing lytic-cycle genes (Staskus *et al.*, 1999).

1.5.2 Detection of HHV-8 in non KS, PEL and MCD affected tissue

1.5.2.1 Skin other than KS

HHV-8 DNA has been detected in normal skin of patients with HIV-associated KS (Gaidano *et al.*, 1996; Corbellino *et al.*, 1996c; Smith *et al.*, 1997b), with classic and endemic KS (Dupin *et al.*, 1995; Lebbe *et al.*, 1997) and in HIV-negative organ transplant patients without KS (Rady *et al.*, 1995). The HHV-8 infection load appears to be lighter in the normal skin than in KS lesions (Dupin *et al.*, 1995).

1.5.2.2 Gastrointestinal tract

Early reports suggested that HHV-8 DNA is rarely detected in throat swabs and sputum of HIV-infected patients with KS (Whitby *et al.*, 1995). Initial studies also suggested that HHV-8 DNA was not present in saliva of patients with HIV-related KS (Ambroziak *et al.*, 1995), but subsequent workers have found HHV-8 DNA not only in oral KS lesions (Jin *et al.*, 1996; Flaitz *et al.*, 1997; Mwakigonja *et al.*, 2007; Pak *et al.*, 2007) but also in the normal oral mucosa of healthy immunocompetent (Duus *et al.*, 2004) and HIV-seropositive individuals (Triantos *et al.*, 2004) and in tonsils and

adenoids of children (Chagas *et al.*, 2006b). HHV-8 has also been detected in gingival biopsies and crevicular fluid samples (Mardirossian *et al.*, 2000) and in oral ulcers (Di Alberti *et al.*, 1997c; Syrjanen *et al.*, 1999) from HIV-seropositive patients, and in saliva of people who were or were not co-infected with HIV and those with or without active KS and can be present in both cellular and cell-free salivary fluid (Blackbourn *et al.*, 1998).

HHV-8 DNA was detected: in mouth rinses from Zimbabwean women with KS (Lampinen *et al.*, 2000); whole unstimulated saliva and oral buccal and lingual scrapes from immunocompromised patients with HIV infection or persons with haematologic malignancies (Triantos *et al.*, 2004); saliva and mouth swabs from HHV-8 seropositive female prostitutes in Kenya (Taylor *et al.*, 2004); and saliva from children with sickle cell disease in Uganda (Mbulaiteye *et al.*, 2004). HHV-8 DNA was also found in saliva and oral swabs of HHV-8 seropositive homosexual American men with and without KS (Koelle *et al.*, 1997; Blackbourn *et al.*, 1998; Pauk *et al.*, 2000; Widmer *et al.*, 2006; Casper *et al.*, 2007). In addition, studies among HIV-infected patients with KS showed that the prevalence of salivary HHV-8 DNA were similar between patients with and without oral KS lesions and HHV-8 was also detected in saliva from patients with resolved KS (Koelle *et al.*, 1997).

Previous findings suggest that major salivary glands may not be important contributors to HHV-8 shedding (Pauk *et al.*, 2000; Corey *et al.*, 2002). However, HHV-8 lytic activation was found to occur as HHV-8-infected keratinocytes differentiate into mature epithelium, which in turn may be responsible for the presence of infectious HHV-8 in saliva (Johnson *et al.*, 2005). Furtherermore, the various oral compartments (e.g. saliva, mucosa) can harbour distinct HHV-8 strains suggesting that different strains may preferentially persist and replicate in different cell types (Beyari *et al.*, 2003).

HHV-8 DNA in oral fluid may be detected at levels independent of peripheral blood mononuclear cell (PBMC) viraemia (Lampinen *et al.*, 2000) and may be intermittent (Koelle *et al.*, 1997; Pauk *et al.*, 2000; Laney *et al.*, 2004; Casper *et al.*, 2007), but individuals who shed virus at one time point are more likely to shed at other times (Laney *et al.*, 2004).

The detection of salivary IgA antibodies with specificities to a latent (ORF 73) and a lytic antigen (ORF K8.1) of HHV-8 in patients with regressed KS indicates anti-HHV-8 antibody activity in saliva and their possible influence on mucosal replication of HHV-8 (Mbopi-Keou *et al.*, 2004).

HHV-8 DNA has also been amplified from duodenal aspirates and intestinal biopsy tissue of HIV-infected individuals (Thomas *et al.*, 1996). However, stool samples could not be found HHV-8 DNA positive in one study (LaDuca *et al.*, 1998).

1.5.2.3 Peripheral blood

HHV-8 exhibits a broad *in vivo* and *in vitro* tropism for blood cells. HHV-8 DNA and transcripts have been identified in peripheral B cells, T cells, monocytes, circulating spindle cells, haemopoietic stem cells, progenitor endothelial cells and fibrocytes (Table 1.4). It has been suggested that HHV-8 infection of lymphocytes and monocytes is responsible for the dissemination of HHV-8, accounting for the multicentric nature of KS (Hengge *et al.*, 2002b).

HHV-8 DNA and transcripts have been identified in PBMCs of patients with KS (Humphrey *et al.*, 1996; Koelle *et al.*, 1997; Viviano *et al.*, 1997; Purvis *et al.*, 1997; LaDuca *et al.*, 1998; Metaxa-Mariatou *et al.*, 2004) and MCD (Kikuta *et al.*, 1997b). The detection rate of HHV-8 in PBMCs of individuals with KS is generally reported to

be more than 50% and appears to be correlated with the stage and extent of KS (Campbell *et al.*, 2000; Camera *et al.*, 2000).

A polyclonal antibody specific for v-IL-6 has been used to demonstrate expression of viral cytokine in HHV-8-infected haematopoetic cells in lymph nodes and in un-induced PEL cell lines (Moore *et al.*, 1996a). The presence of circular and linear HHV-8 genomes in PBMCs was reported in one study, reflecting the presence of both latently and productively infected cells (Decker *et al.*, 1996).

HHV-8 plasma viraemia has been suggested to be an important event in KS pathogenesis and detectable HHV-8 DNA in serum or plasma is usually associated with active disease (Harrington *et al.*, 1996; Tedeschi *et al.*, 2001; Campbell *et al.*, 2003).

Table 1.4 HHV-8 in peripheral blood sub-populations

Cell type	CD Marker	Details	Reference
B cells	CD19	3 HIV-infected patients with KS	(Ambroziak *et al.*, 1995)
	CD19	1 patient with post-transplant KS and 1 patient with AIDS-KS	(Harrington *et al.*, 1996)
	CD19	B cells infected with HHV-8 from BC-1 cell line	(Mesri *et al.*, 1996)
	CD19	2 HIV negative patients with MCD and 1 patient with localized Castleman's disease (LCD)	(Kikuta *et al.*, 1997b)
	CD19	9 patients with and 1 without KS	(Henry *et al.*, 1999)
	CD19	3 HIV-infected men with and without KS	(Monini *et al.*, 1999)
	CD19	1 patient with stable and 2 patients with progressive KS	(Pellet *et al.*, 2006)
T cells	CD3	1 patient with post-transplant KS and 1 patient with AIDS-KS	(Harrington *et al.*, 1996)
	CD2	2 HIV negative patients with MCD and 1 patient with LCD	(Kikuta *et al.*, 1997b)
	CD2	4 patients with AIDS-KS	(Henry *et al.*, 1999)
	CD8	1 patient with AIDS-KS	(Sirianni *et al.*, 1997b)
	N/S	1 late stage AIDS-KS patient	(Monini *et al.*, 1999)
	CD2	1 patient with progressive KS	(Pellet *et al.*, 2006)
Monocytes	CD14	1 patient with classic KS and 2 patients with AIDS-KS	(Henry *et al.*, 1999)
	N/S	5 HIV infected men with and without KS	(Monini *et al.*, 1999)
	CD14	1 patient with stable and 1 patient with progressive KS	(Pellet *et al.*, 2006)
Circulating spindle cells	N/S	3 patients with AIDS-KS, 2 patients with post-transplant KS and 11 with classic KS	(Sirianni *et al.*, 1997a)
	CD31	1 HIV-1-infected gay man without KS	(Kumar *et al.*, 2007)
Haemopoietic stem cells / progenitor endothelial cells, fibrocytes	CD34	1 patient with classic KS and 8 patients with AIDS-KS	(Henry *et al.*, 1999)
	CD34	1 patients with stable and 3 patients with progressive KS	(Pellet *et al.*, 2006)
All haemopoietic cells	CD45	38 HIV-1-infected patients without KS and 9 blood donors	(Kumar *et al.*, 2007)

N/S: not specified

1.5.2.4 Genital tissue and fluids

Several studies suggest the presence of HHV-8 in prostatic tissues of some HIV-infected men with or without KS (Corbellino *et al.*, 1996c; Staskus *et al.*, 1997; Diamond *et al.*, 1998; Montgomery *et al.*, 2006). *In situ* hybridization (Staskus et al., 1997) and immunohistochemistry (Montgomery *et al.*, 2006) studies showed that HHV-8 gene expression in prostatic glandular epithelium is common in people without KS, lending support to the supposition that the virus is widely disseminated in the healthy male population. However, studies by PCR have not yet identified prostatic tissue from men without KS to be infected with HHV-8 (Tasaka *et al.*, 1996; Corbellino *et al.*, 1996a; Rubin *et al.*, 1998).

HHV-8 was found to be preferentially detected in semen rather than spermatocytes, suggesting secretion into seminal fluids (Monini *et al.*, 1996b; Howard *et al.*, 1997), but it cannot be excluded that HHV-8-infected mononuclear cells occasionally represent the source of HHV-8 in semen. It has been detected in some semen samples from HIV-infected patients with or without KS, but the reported detection rates in the United Kingdom and the United States vary from 0% (of only four samples tested) to 33% (Ambroziak *et al.*, 1995; Marchioli *et al.*, 1996; Gupta *et al.*, 1996; Howard *et al.*, 1997). The results obtained in semen samples from healthy, HIV-seronegative donors are even more controversial. A high HHV-8 detection rate was reported from Italy, where it was initially reported to be 91% positive (Monini *et al.*, 1996b), but later to be 23% positive (Monini *et al.*, 1996a) similar to that detected in semen donors from the United States (Lin *et al.*, 1995). On the other hand, no positive samples were found among 115 semen donors in the United Kingdom (Howard *et al.*, 1997) or in 20 in Milan, Italy (Corbellino *et al.*, 1996a). Some of these discrepant results probably reflect regional differences in HHV-8 prevalence, or selection of semen donors, or both.

The genital tissues of women also harbour HHV-8, with the virus found in cervical and vaginal swabs from HIV positive African women (Lampinen *et al.*, 2000; Taylor *et al.*, 2004). However, HHV-8 has so far not been found in vulval mucosa or cervical malignancies of females without a history of HIV disease (Tasaka *et al.*, 1997).

1.5.2.5 Respiratory tract

HHV-8 DNA has been detected in nasal secretions (Blackbourn *et al.*, 1998) and in bronchoalveolar lavage (BAL) fluid from severely immunocompromised HIV-infected patients with pulmonary symptoms. HHV-8 DNA in BAL fluid may indicate the presence of subclinical KS (Tarp *et al.*, 2001).

1.5.2.6 Neuronal tissue

There are limited data available, as yet, about the possible neurotropism of HHV-8. It has been suggested in one report that dorsal root ganglia in patients with AIDS and KS harbour viral DNA (Corbellino *et al.*, 1996b). HHV-8 has also been detected in the cerebrospinal fluid from HIV-infected patients (Brink *et al.*, 1998). Furthermore, HHV-8 DNA has been demonstrated in cerebral tissue from patients with multiple sclerosis and also in healthy brain tissue (Merelli *et al.*, 1997; Chan *et al.*, 2000).

1.5.2.7 Urine

Urine has recently been identified as another body fluid into which HHV-8 can shed. HHV-8 was detected in urine samples from 6.4% of Malawian KS patients (Beyari *et al.*, 2004),11% of Brazilian KS patients and 28% of HIV-1-infected patients without KS (Santos-Fortuna and Caterino-de-Araujo, 2005).

1.5.2.8 In vitro studies

In vitro, HHV-8 has been shown to infect a variety of human and animal cells, such as human B cells, human epithelial cells, human endothelial cells, human foreskin fibroblast cells, human carcinoma cells (bladder, prostate, lung, and squamous), owl monkey kidney cells, and baby hamster kidney (BHK-21) cells (Flore *et al.*, 1998; Renne *et al.*, 1998; Moses *et al.*, 1999; Vieira *et al.*, 2001). Indeed, Bechtel *et al.* (2003) confirmed that most adherent cell lines, irrespective of species of origin or tissue lineage, are permissive for HHV-8 viral entry and the establishment of latency (Bechtel *et al.*, 2003). This indicates a wide distribution of receptors, possibly integrins, capable of binding to the virus (Akula *et al.*, 2002).

1.6 Routes of HHV-8 transmission

The primary mode of HHV-8 transmission remains unresolved. Extensive evidence exists for both sexual transmission (Lin *et al.*, 1995; Howard *et al.*, 1997; Casper *et al.*, 2002) and non-sexual transmission (Koelle *et al.*, 1997; Vieira *et al.*, 1997; Blackbourn *et al.*, 1998; Lampinen *et al.*, 2000; Pauk *et al.*, 2000; Duus *et al.*, 2004; Triantos *et al.*, 2004; Widmer *et al.*, 2006; Casper *et al.*, 2007).

1.6.1 Sexual transmission

Transmission of HHV-8 is primarily sexual among homosexual men (Vieira *et al.*, 1997; Melbye *et al.*, 1998; Martin *et al.*, 1998; Dukers *et al.*, 2000; Casper *et al.*, 2002; Martro *et al.*, 2007). HHV-8 infection is more common among people practising high-risk sexual behaviours (Casper *et al.*, 2006) and those attending sexually transmitted disease clinics than blood donors (Kedes *et al.*, 1996; Simpson *et al.*, 1996; Lennette *et al.*, 1996). An association of HHV-8 and HSV-2 infections was found in HIV-infected US military men (Crum *et al.*, 2003) and Italian prison inmates (Sarmati *et al.*, 2007), further suggesting sexual HHV-8 transmission. Further evidence comes from the finding of nearly identical HHV-8 strains in Cuban HIV

couples discordant for KS (Kouri *et al.*, 2007).

However, the specific mechanisms of transmission remain controversial (Martro *et al.*, 2007). Some of the early studies on the relationship between KS and sexual practices report an association between oral-anal sex and AIDS KS (Grulich and Kaldor, 1996; Vieira *et al.*, 1997). One study associated specific sexual techniques to serococonversion (Dukers *et al.*, 2000), implicating recent oral-genital sex as a strong risk factor. Cross-sectional studies have demonstrated an association between the presence of HHV-8 antibodies and anal-genital sex, oral-anal sex, and deep kissing with an HIV-1-positive partner (Melbye *et al.*, 1998; Grulich *et al.*, 1999; Pauk *et al.*, 2000).

On the assumption that HHV-8 can be sexually transmitted, the virus would need to be shed at high titres in semen. Indeed, HHV-8 DNA has been detected in prostate tissue biopsies of HIV-seropositive men with and without KS (Corbellino *et al.*, 1996c; Staskus *et al.*, 1997; Diamond *et al.*, 1998; Montgomery *et al.*, 2006), indicating that HHV-8 can be shed from the prostate into semen.

Furthermore, longitudinal studies of viral shedding have shown that HHV-8 is not substantially shed in semen or rectal tissue, but instead is persistently found in the oral cavities of many individuals (Pauk *et al.*, 2000; Corey *et al.*, 2002). Infectious HHV-8 has now been detected in high titres in saliva of HHV-8-seropositive individuals (Koelle *et al.*, 1997; Vieira *et al.*, 1997; Blackbourn *et al.*, 1998; Pauk *et al.*, 2000). Since oral-genital sex is a common practice among homosexual men, as is deep kissing (Myers *et al.*, 1992; Pauk *et al.*, 2000; Dukers *et al.*, 2000; Crum *et al.*, 2003), saliva rather than semen is now considered as a significant vehicle of HHV-8 transmission.

Among heterosexuals, correlations with sexual transmission have not been consistently maintained (Smith *et al.*, 1999; Wawer *et al.*, 2001). Heterosexual transmission is not likely to be a major route and seems to require frequent sexual exposure to occur. HHV-8 prevalence is elevated in heterosexuals who report consorting with multiple sexual partners (Bestetti *et al.*, 1998; Tedeschi *et al.*, 2000; de Sanjose *et al.*, 2002).

1.6.2 Non sexual transmission

1.6.2.1 Horizontal /Household

There is gathering evidence that sexual transmission is not the primary route of infection with HHV-8 in countries of high prevalence. In Africa, HHV-8 seroprevalence is relatively high in children, with seroprevalence reaching adult levels before adolescence (Mayama *et al.*, 1998; Gessain *et al.*, 1999; Plancoulaine *et al.*, 2000). These data suggest transmission via close, non-sexual routes. Intrafamilial transmission has been suggested by the presence of HHV-8 DNA in households with high density or crowding (Mbulaiteye *et al.*, 2004; Brown *et al.*, 2005) and by the finding that children are more likely to be HHV-8 seropositive if they have mothers or siblings who are seropositive (Plancoulaine *et al.*, 2000; Mbulaiteye *et al.*, 2005; Simonart, 2006). Clustering of HHV-8 seroprevalence between spouses, children, and siblings was demonstrated in a Sardinian study population (Angeloni *et al.*, 1998).

In Middle Eastern countries, evidence of HHV-8 infection in children also supports non-sexual transmission. In Egyptian children, HHV-8 seroprevalence exceeded 50% in children older than 6 yr and increased steadily up to 10 yr, thereafter stabilising (Andreoni *et al.*, 1999). In these children, HHV-8 primary infection has been positively associated with close contact with at least two other children in the community, pointing to saliva as an important route of paediatric infection (Andreoni *et al.*, 2002).

Moreover, for Israeli children, HHV-8 seroprevalence also rises with age. Davidovici *et al.* (2001) reported that HHV-8 infection in children of a seropositive mother was influenced by the antibody titre of the mother, but was unassociated with the HHV-8-infected father.

Gessain *et al.* (1999) found HHV-8 infection to be common among children in Cameroon. Seroprevalence steadily increased from 27% at age of 4 yr to 39% in the 12-14 yr group, to above 48% in children over 15 yr, approaching the level of HHV-8 infection in adults. Plancoulaine *et al.* (2000) studied a population of villagers of African origin in French Guiana and showed that the HHV-8 seroprevalence was 15% by age of 15 yr and rising sharply to about 30% after 40 yr; they provided evidence to implicate horizontal familial transmission between children and their mother but not their fathers, and between children and their siblings. A similar study conducted in rural Tanzania found associations between the HHV-8 serostatus of spouses and between the serostatus of a father and that of his children (Mbulaiteye *et al.*, 2003b).

In a study of KS patients and their first-degree relatives in Malawi (Cook *et al.*, 2002a; Cook *et al.*, 2002b), and between Ugandan mother-child pairs (Mbulaiteye *et al.*, 2006), both identical and non-identical HHV-8 sub-genomic sequences were observed between family members, suggesting transmission of HHV-8 along both intra- and extra-familial transmission routes.

A recent study in sub-Saharan Africa demonstrated the absence of HHV-8 DNA in the breast milk of seropositive mothers, suggesting that contact with breast milk is not a likely source of horizontal transmission of virus to infants (Brayfield *et al.*, 2004). However, a study conducted by Dedicoat and co-workers in South Africa showed presence of HHV-8 DNA in 12 out of 43 breast milk samples (Dedicoat *et al.*, 2004). A positive correlation with hepatitis B infection suggests that HHV-8 is transmitted

horizontally in conditions of close contact and crowding (Mayama *et al.*, 1998). Although the precise mode of non-sexual horizontal transmission has not been clearly established, the relatively frequent carriage of HHV-8 in oral fluids suggests that transmission may occur via shedding from the oral cavity.

1.6.2.2 Oral

There have been an increasing number of studies whose results support the hypothesis that the mouth and oropharynx are dominant sites of HHV-8 shedding, and saliva as an important vehicle of HHV-8 transmission (Koelle *et al.*, 1997; Gessain *et al.*, 1997; Di Alberti *et al.*, 1997a; Blackbourn *et al.*, 1998; Plancoulaine *et al.*, 2000; Lampinen *et al.*, 2000; Cook *et al.*, 2002a; Beyari *et al.*, 2003; Taylor *et al.*, 2004; Duus *et al.*, 2004; Triantos *et al.*, 2004; Mbulaiteye *et al.*, 2004; Mbopi-Keou *et al.*, 2004; Martro *et al.*, 2004; Widmer *et al.*, 2006; Chagas *et al.*, 2006a; Casper *et al.*, 2007; Pak *et al.*, 2007; de Souza *et al.*, 2007b). In addition, the high prevalence of infection before adolescence in endemic areas and the higher frequency of the detection of HHV-8 in saliva compared with semen (Pauk *et al.*, 2000) provide indirect evidence of non-sexual transmission.

In U.S HIV-infected homosexual men, increased HHV-8 oropharyngeal shedding has been observed to correlate with increased CD4 count, inflammation in the oral cavity and absence of antiretroviral therapy (Casper *et al.*, 2004). However, Widmer *et al.* (2006) reported the detection of oral HHV-8 in the Swiss HIV-infected Cohort Study patients with or without KS not to be influenced by patients HIV immune status, but did observe a trend toward higher detection in patients with KS. They also reported that oral HHV-8 shedding had no predictive value either for the development of KS lesions or for survival.

Although the virus has been detected in saliva, the identities of the cell types

harbouring HHV-8 *in vivo* in the oral cavity and producing virus particles is unclear. Corey *et al.* (2002) compared HHV-8 DNA content in whole saliva versus parotid duct secretions and found HHV-8 to be absent or markedly lower in parotid fluid than whole saliva; minor salivary gland biopsies performed in 4 HHV-8-seropositive persons who shed HHV-8 in their saliva showed no evidence of HHV-8 in the biopsy tissues using *in situ* hybridization. However, HHV-8 DNA has been previously found in buccal cells of healthy immunocompetent individuals (Duus *et al.*, 2004) and buccal and palatal exfoliates of Malawian KS patients and their relatives (Cook *et al.*, 2002a; Cook *et al.*, 2002b; Beyari *et al.*, 2003). In addition, the ability of the virus to infect primary human keratinocytes has also been shown (Cerimele *et al.*, 2001; Duus *et al.*, 2004).

A model of reactivation and shedding similar to EBV has been suggested in which HHV-8 shed into the mouth originates from activation in latently infected B cells infecting the epithelial cells that line the lymph nodes (Sixbey *et al.*, 1984; Bigoni *et al.*, 1996; Ivarsson *et al.*, 1999). In palatine tonsils of two HIV-infected HHV-8-seropositive persons, the lytic gene products of HHV-8 in B cells within the germinal centre and the epithelial lining of tonsil could be detected, suggesting that lytic replication of HHV-8 in B cells within the germinal centre may transfer HHV-8 to the epithelial surface either by its migration to the surface epithelium or by contiguous infection (Corey *et al.*, 2002). An alternative model suggests that HHV-8 persists and replicates in oral epithelial cells of KS patients or even of healthy individuals, and that these are the principal source of virus in saliva (Vieira *et al.*, 1997; Duus *et al.*, 2004).

A study in South Africa reported that HHV-8 DNA was detectable in 145 of 978 maternal saliva samples (mean virus load: 488,450 copies/ml), suggesting saliva as a significant vehicle of mother-to-child transmission (Dedicoat *et al.*, 2004). HHV-8 may therefore be transferred from mother to child via premastication of food and use of

shared saliva-contaminated items. Other than close contact and the use of shared items (spoons, plates, etc.) in an endemic area like Africa, behavioural practices associated with saliva exchange in sub-Saharan Africa may play a role in transmission of HHV-8. The use of saliva in healing medical practices, religious initiation or ritual practices, and feeding practices have been implicated (Wojcicki, 2003; Wojcicki *et al.*, 2007). However, close contact and sharing amongst patients in renal care units has not yet been fully investigated.

1.6.2.3 Vertical

Whether HHV-8 infection may be transmitted from mother to child is still unclear, with studies showing conflicting results. A study in South Africa suggests a rate of vertical transmission as high as 30% (Bourboulia *et al.*, 1998). Other studies showed that transmission appears to increase with increasing maternal antibody titres (Sitas *et al.*, 1999b; Lisco *et al.*, 2006). However, It has been demonstrated that seropositive infants born to seropositive mothers, later become seronegative by 24 months (Calabro *et al.*, 2000), suggesting that positive results are due to the transfer of maternal antibodies (Gessain *et al.*, 1999; Calabro *et al.*, 2000). On the other hand, Mantina *et al.* (2001) showed detection of viral DNA in PBMCs at the time of delivery for 2 of 89 infants born to HHV-8-seropositive mothers, which suggests that *in utero* infection occurs but rarely (Mantina *et al.*, 2001). A study in Zambia showed no association between the HHV-8 infection rates among infants and their respective mothers (Brayfield *et al.*, 2003). A more recent study in Italy also suggests that vertical transmission of HHV-8 is either unlikely or very rare (Sarmati *et al.*, 2004).

1.6.2.4 Blood

HHV-8 also appears to be transmissible, although less frequently, via exposure to blood. Some studies suggest that blood transfusion is associated with a very small risk of HHV-8 transmission (Operskalski *et al.*, 1997; Engels *et al.*, 1999; Challine *et*

al., 2001; Pellett *et al.*, 2003; Mbulaiteye *et al.*, 2003a; Garcia-Astudillo and Leyva-Cobian, 2006), with the paucity of B lymphocytes, accompanying most types of transfusion, thought to contribute to this low transmission risk (Engels *et al.*, 1999). Furthermore, previous studies have shown HHV-8 infection to be relatively rare in frequently transfused groups, such as haemophiliacs (Operskalski *et al.*, 1997) and thalassemia or sickle cell anemia patients (Lefrere *et al.*, 1997). In addition, low-prevalence areas generally show a similar prevalence of HHV-8 between injecting drug users (IDUs) and the general population (Renwick *et al.*, 2002).

On the other hand, a number of studies (Velez-Garcia *et al.*, 1985; Bendsoe *et al.*, 1990; Padilla *et al.*, 1990; Aboulafia *et al.*, 1991; Belec *et al.*, 1998; Baillargeon *et al.*, 2001; Enbom *et al.*, 2002) implicating that transfusion and parenteral exposure are risk factors for KS have long existed. Some studies, which were performed in intermediate or high endemic populations, have shown that the HHV-8 prevalence is somewhat higher among IDUs compared with the general population (Cannon *et al.*, 2001; Sosa *et al.*, 2001; Parisi *et al.*, 2002). Furthermore, the recovery of infectious HHV-8 from a seropositive healthy North American blood donor (Blackbourn *et al.*, 1997) and HHV-8 transmission by blood transfusion in the US (Dollard *et al.*, 2005) and Uganda (Hladik *et al.*, 2006) have been reported. Selective screening for HHV-8 during cellular therapy (Lefrere *et al.*, 2007) and blood transfusion in immunocompromised populations (Baillargeon *et al.*, 2001; Hladik *et al.*, 2006; Moore *et al.*, 2007) have been suggested.

1.6.3 Haemodialysis

HHV-8 transmission in the haemodialysis setting has been a controversial issue. Some studies have found a higher prevalence of HHV-8 antibodies in hemodialysis patients than in the normal blood donors (Qunibi *et al.*, 1998; Regamey *et al.*, 1998; Hsu *et al.*, 2002), or a higher risk of occupational HHV-8 infection among medical staff

caring for HHV-8 risk group patients including patients on haemodialysis (Gartner *et al.*, 2003), suggesting that this transmission route may be possible. Other studies comparing HHV-8 antibodies in haemodialysis patients with that in the general population (Luppi *et al.*, 1999; Cattani *et al.*, 2001; Di Stefano *et al.*, 2006) and analysing patterns of change in HHV-8 serostatus (Zavitsanou *et al.*, 2006) suggest HHV-8 transmission in the haemodialysis setting to be uncommon .

1.6.4 Organ transplantation

Studies from various groups, which generate findings based on serology and molecular tracking of infection, have shown that organ-related transmission of HHV-8, especially in regions where the virus is not endemic, is more common than previously thought (Parravicini *et al.*, 1997; Regamey *et al.*, 1998; Luppi *et al.*, 2000a; Luppi *et al.*, 2000b; Sarid *et al.*, 2001; Cattani *et al.*, 2001; Kapelushnik *et al.*, 2001; Munoz *et al.*, 2002; Barozzi *et al.*, 2003; Collart *et al.*, 2004; Marcelin *et al.*, 2004; Becuwe *et al.*, 2005) and that the risk of KS could be exceedingly high when the organ donor and recipient are both HHV-8 seropositive (Bergallo *et al.*, 2007). The development of the neoplasm may reflect a decreased capacity of the host to destroy cells harbouring latent HHV-8 because of continuous immunosuppression (Bottalico *et al.*, 1997). Accordingly, systematic screening for HHV-8 antibodies in recipients and organ donors (Milliancourt *et al.*, 2001), screening of grafts for HHV-8, use of post-transplantation HHV-8 diagnostics (Marcelin *et al.*, 2007), pre-emptive interventions in HHV-8 infected individuals with antivirals (Luppi *et al.*, 2003; Garcia-Astudillo and Leyva-Cobian, 2006), and the use of donor-derived HHV-8-specific T cells for the control of post-transplant KS (Barozzi *et al.*, 2003) have been advocated.

However, in regions where the virus is endemic, such as Italy, most recipients of renal allografts are seropositive for HHV-8 before transplantation, but none show clinical manifestations of KS until immunosuppressive therapy begins, which suggests that

post-transplant KS is caused by virus reactivation (Parravicini *et al.*, 1997; Cattani *et al.*, 2001; Weigert *et al.*, 2004; Bergallo *et al.*, 2007). Post-transplant primary virus transmission from an outside source rather than donor-to-recipient transmission has also been suggested (Jenkins *et al.*, 2002; Bergallo *et al.*, 2007). Primary infection after transplantation has been suggested to pose a greater risk of complications than does reactivation of pre-existing virus (Sarid *et al.*, 2001; Marcelin *et al.*, 2004) and early post transplant KS seems to be associated with a poorer outcome (Cozar *et al.*, 1990). Therefore the distinction between HHV-8 reactivation and primary infection may be important for predicting outcome in regard to KS development.

1.7 Primary HHV-8 infection

Clinical manifestations of primary infection with HHV-8 have been described but are not thought to be associated with significant concurrent morbidity in immunocompetent populations (Kasolo *et al.*, 1997; Kikuta *et al.*, 1997a; Plancoulaine *et al.*, 2000; Luppi *et al.*, 2000b; Andreoni *et al.*, 2002). In immunocompetent children, primary HHV-8 infection may be asymptomatic or associated with a febrile maculopapular rash (Plancoulaine *et al.*, 2000; Andreoni *et al.*, 2002; Gilden *et al.*, 2007).

The outcome of primary HHV-8 infection in immunosuppressed hosts can be variable, and it is also more difficult to directly attribute symptoms to HHV-8 rather than the immunosuppression. However, bone marrow failure, splenomegaly and fever have been reported in adult patients upon organ transplantation (Luppi *et al.*, 2000b) and transient angiolymphoid hyperplasia was found to occur as part of an HHV-8-seroconversion syndrome in an HIV-infected adult (Plancoulaine *et al.*, 2000). Furthermore, a case report of an HIV-1-seropositive male noted HHV-8 seroconversion five weeks prior to the sudden onset of symptoms, which included fever, arthralgia, cervical lymphadenopathy, and splenomegaly. PCR of the cervical

mass specimen revealed HHV-8 DNA, vascular hyperplasia, and "intense" activation and proliferation of B cells which was negative for EBV gene expression. The febrile episode was prolonged but resolved spontaneously within 2 months (Oksenhendler *et al.*, 1998a). It has also been reported that HIV-positive patients with evidence of subsequent HHV-8 seroconversion showed a greater relative risk for progression to KS than did those who had seroconverted for HHV-8 prior to HIV-1 infection (Goudsmit *et al.*, 2000). Hence it appears that primary HHV-8 infection has a higher morbidity in those who are HIV-1-infected than in those who reactivate a previous latent HHV-8 infection, as did primary infection after transplantation.

1.8 HHV-8 associated diseases

1.8.1 KS

KS is a vascular tumour that was brought to the attention of the medical community over a century ago in 1872 by the report of purple-coloured nodular skin lesions in five elderly men by Moritz Kaposi. Four epidemiological forms of KS based on clinical and epidemiological differences are now recognised: classic KS, endemic-HIV-negative KS, iatrogenic KS, and HIV-associated or epidemic KS. In 1994, Chang and co-workers reported the identification of fragments of herpesvirus-like DNA in AIDS-KS biopsy samples (Chang *et al.*, 1994). HHV-8 is now accepted as the etiological agent for all the various forms of KS.

1.8.1.1 Clinical features and epidemiology

The four distinct epidemiological forms of KS are characterized by different demographic parameters, risk factors, clinical presentation, and outcomes (Cohen *et al.*, 2005). For many years, KS was thought to be a lesion that affected predominantly elderly patients of Mediterranean, East European, Arabic or Jewish ancestry (Franceschi and Geddes, 1995; Antman and Chang, 2000; Cohen *et al.*, 2005); this form is known as classic KS. The incidence peaks after the 6th decade of life

(DiGiovanna and Safai, 1981). It affects the extremities, is generally indolent and is more common in men than women (sex ratio estimated to be from 3:1 to 15:1) (Franceschi and Geddes, 1995; Hengge *et al.*, 2002a). Classic KS runs a chronic course and rarely metastasises. Patients survive an average of 10-15 yr before dying of unrelated causes (Cohen *et al.*, 2005). Complications include lymphoedema, hyperkeratosis and other neoplasms such as non-Hodgkin's lymphoma and cutanous malignant melanoma (Friedman-Birnbaum *et al.*, 1990; Iscovich *et al.*, 1999).

KS was already present in equatorial Africa for many decades preceding the HIV epidemic (Oettle, 1962). A 1971 report showed that KS accounted for 9% of all reported cancers in Ugandan males (Taylor *et al.*, 1971). A study published in 1993 showed that KS in HIV-negative and HIV-positive patients accounted for approximately 50% of all tumours diagnosed in men in some central African countries (Wabinga *et al.*, 1993). African or endemic KS is usually more aggressive than the classic form of KS (D'Oliveira and Torres, 1972). 'Endemic' KS affects two main age groups: young men with an average age of 35 yr and children with an average age of 3 yr (Wabinga *et al.*, 1993). In adults, it presents as a benign nodular cutaneous disease or as a florid mucocutaneous oral visceral disease. In young children, it can be aggressive, with localised cutaneous disease progressing to invade adjacent soft tissues and bones or rapidly disseminating to lymph nodes and visceral organs.

The third form of KS has been described in iatrogenically immunosuppressed organ transplant recipients and in a wide spectrum of patients receiving chronic immunosuppressive therapy (Penn, 1983; Qunibi *et al.*, 1988; Farge, 1993; Szende *et al.*, 1997; Sachsenberg-Studer *et al.*, 1999; Garcia-Astudillo and Leyva-Cobian, 2006). Iatrogenic KS may be aggressive, involving lymph nodes, the mucosa and the viscera but remission can occur spontaneously after discontinuation of immunosuppressive therapy. However, there is a high risk of graft loss (Brooks,

1986; Veroux *et al.*, 2004; Moray *et al.*, 2004; Huang *et al.*, 2004).

The fourth variant of KS, the 'epidemic' type, was identified in the early 1980s. It heralded the AIDS epidemic (Hymes *et al.*, 1981), and was later recognised as an AIDS-defining condition in HIV-infected individuals, the most common neoplasm occurring in patients with AIDS (Beral *et al.*, 1990; Rabkin *et al.*, 1995) and the first clinical manifestation in about one quarter of AIDS patients (Goedert, 2000). The overall risk of KS in AIDS patients is estimated to be more than 20,000 times greater than that of the general population and 300 times that of other immunosuppressed patients (Beral *et al.*, 1990). Striking differences in risks of acquiring AIDS-KS were observed between different HIV transmission groups, with the risk in homosexual men being more than men with haemophilia and the risk in women acquiring HIV from bisexual men being higher than heterosexual intravenous drug users (Haverkos and Drotman, 1985; Beral *et al.*, 1990; Serraino *et al.*, 1995; Goedert, 2000). Anti-retroviral therapy (ART) has been shown to greatly reduce the incidence of KS in HIV disease (Gill *et al.*, 2002; Cattelan *et al.*, 2005; Grabar *et al.*, 2006; Martro *et al.*, 2007). On the other hand, proliferation of KS as part of ART-associated immune reconstitution inflammatory syndrome has occasionally been observed (Weir and Wansbrough-Jones, 1997; Connick *et al.*, 2004; Nathan, 2007).

Epidemic KS is typically lymphoadenopathic and tends to involve the viscera and mucosa as well as the skin. It is commonly multifocal, symmetrical and frequently on the upper body, head and neck (Hengge *et al.*, 2002a; Wamburu *et al.*, 2006) and may involve the lower limbs (Onunu *et al.*, 2007). It may evolve quickly, both in local progression of lesions to tumours and in visceral dissemination, leading to organ dysfunction and high mortality (Hengge *et al.*, 2002a; Wamburu *et al.*, 2006). Before ART became available, oral KS was among the first clinical manifestations of AIDS (Silverman *et al.*, 1986; Schwartz, 2004), the oral KS lesion appearing as a red-

purple macule, an ulcer, or as a nodule or mass. Intraoral KS occurs on the heavily keratinized mucosa, the palate being the site of predilection in more than 90% of reported cases; other affected sites include the gingiva, tongue and the buccal mucosa (Silverman *et al.*, 1986; Epstein and Scully, 1991; Glick, 1992; Nichols *et al.*, 1993). Intraosseous involvement of the jaws with KS has been reported (Langford *et al.*, 1991; Nichols *et al.*, 1995; Noel *et al.*, 2007). The oral lesions may result in pain, dysphagia, difficulty with mastication, bleeding, and may be cosmetically displeasing (Epstein and Scully, 1991; Nichols *et al.*, 1993; Flaitz *et al.*, 1997). A female predisposition to oral KS has been observed recently in Tanzania, this being associated with HIV/AIDS and advanced nodular histopathology (Mwakigonja *et al.*, 2007).

1.8.1.2 Histogenesis of KS

The histopathology of the 4 clinico-epidemiological forms of KS is essentially the same, with characteristic changes related to stage in the development of the KS tumour. KS is a complex, multifocal, highly and abnormally vascularized tumour-like lesion which develops from early inflammatory stages of patch/plaque to late, nodular tumours composed predominantly of spindle cells (Pyakurel *et al.*, 2007). In the "patch" stage the histological picture is that of endothelial-lined spaces surrounding normal blood vessels; there is often a variable degree of inflammatory infiltrate. The "plaque" stage is characterised by an increased growth of spindle-shaped vascular processes in the dermis, along with the formation of slit-like vascular spaces filled with erythrocytes. In the late, "nodular" stage, the lesion is comprised primarily of spindle shaped cells arranged in large sheets, some of which are undergoing mitosis, with slit-like vascular spaces and haemosiderin pigmentation (Cockerell, 1991).

KS represents a unique variant of tumour growth with continuous recruitment of tumour precursor cells as well as proliferation and decreased apoptosis of spindle

cells (Pyakurel *et al.*, 2006). KS may be a reactive proliferation rather than a true malignancy with metastatic dissemination (Duprez *et al.*, 2007). The spindle cells are thought to be the tumour cells that form the bulk of established KS but their histogenesis is unknown. The majority of spindle cells express lymphatic endothelial cell markers, including CD31 and CD34 markers. However, some express markers of smooth muscle cells, macrophages and dendritic cells (Nickoloff and Griffiths, 1989; Sturzl *et al.*, 1992), suggesting that spindle cells represent a heterogeneous population of cells, or cells that arise from a pluripotent mesenchymal precursor cell. All KS spindle cells express vascular endothelial growth factor receptor 3 (VEGFR-3), which is usually expressed only by lymphatic endothelium and by neoangiogenic vessels, but not by mature vascular endothelial cells, indicating that KS spindle cells may be of the endothelial lineage that can differentiate into lymphatic cells (Dupin *et al.*, 1999; Carroll *et al.*, 2004; Pyakurel *et al.*, 2006).

KS, like most tumours, produces and responds to cytokines. Spindle cells or infiltrating CD8+ lymphocytes and macrophages express high levels of IL-6, basic fibroblast growth factor (bFGF), gamma interferon, and a variety of other cytokines (Ensoli *et al.*, 1989; Miles *et al.*, 1990; Sirianni *et al.*, 1998; Fiorelli *et al.*, 1998; Dupin *et al.*, 1999). Biologically active IL-6 and bFGF can promote spindle cell proliferation. Gamma-IFN can induce KS-like tumour formation in nude mice and these tumours in turn produce bFGF (Ensoli *et al.*, 1989; Fiorelli *et al.*, 1998).

HIV-1-associated KS exhibits a more aggressive clinical course than the other types of KS. Therefore, it has been proposed that HIV-1 gene products may positively influence KS development (Ensoli *et al.*, 1994). The *tat* gene in HIV-1 codes for an early trans-activator protein (Tat) essential for HIV-1 viral replication (Fisher *et al.*, 1986). It is released into the extracellular fluid during acute HIV infection, can promote spindle cell growth *in vitro* (Ensoli *et al.*, 1993) and induces an angiogenic

phenotype in nude mice (Vogel *et al.*, 1988). Normal endothelial cells display a spindle cell-like morphology when exposed to Tat in the presence of other cytokines (e.g. IL-1, tumor necrosis factor, and gamma interferon) in culture. Indeed, it has been proposed that these cytokines, whose production is up-regulated in persons with HIV-1 infection, work synergistically with Tat in KS development (Barillari *et al.*, 1992). Studies have indicated that Tat may influence the specific tissue distribution of KS (nose, oral and genital mucosa) observed in HIV-infected individuals (Prakash *et al.*, 2000).

1.8.1.3 Establishing the causal role of HHV-8 in KS

Four observations link HHV-8 causally to the aetiopathogenesis of KS:

i) HHV-8 DNA is present, by PCR in all epidemiological forms of KS, in all fresh biopsies tested and in the vast majority of paraffin-embedded material (Moore and Chang, 1995; Ambroziak *et al.*, 1995; Schalling *et al.*, 1995; Boshoff *et al.*, 1995b; Chang and Moore, 1996; Kasolo *et al.*, 1997; Penn, 1997). The virus is found in HIV-positive and HIV-negative patients with KS (Ambroziak *et al.*, 1995). To strengthen the molecular epidemiological association between HHV-8 and KS further, it was demonstrated by PCR *in situ* hybridization, RNA *in situ* hybridization and immunohistochemistry that HHV-8 is present in spindle cells in nearly all KS lesions (Boshoff *et al.*, 1995b; Li *et al.*, 1996; Sturzl *et al.*, 1997; Rainbow *et al.*, 1997; Staskus *et al.*, 1997; Dupin *et al.*, 1999; Kellam *et al.*, 1999; Sturzl *et al.*, 1999).

ii) The detection of HHV-8 DNA in PBMCs significantly correlates with the risk of developing classic KS (Brown *et al.*, 2006). In addition, HHV-8 sequence may be detected by PCR in the peripheral blood of HIV-positive individuals before the onset of KS lesions (Whitby *et al.*, 1995; Lefrere *et al.*, 1996; Moore *et al.*, 1996c). In individuals without KS or HIV infection, the HHV-8 detection rate in PBMCs is more variable.

iii) Seroprevalence studies indicate that in populations at risk of developing KS, there was a higher prevalence of HHV-8 infection (to be discussed in more detail later). Epidemiological prospective cohort studies have shown that among HIV infected gay men seroconversion to HHV-8 is strongly predictive of the future development of KS (Melbye *et al.*, 1998; Renwick *et al.*, 1998; O'Brien *et al.*, 1999; Jacobson *et al.*, 2000).

iv) HHV-8 encodes several genes that can stimulate cellular proliferation and migration, prevent apoptosis, and counter the host immune response (as described in Section 1.3), transforming cells in susceptible hosts to a malignant phenotype, thereby contributing to the establishment and progression of KS (Moore and Chang, 2003; McAllister and Moses, 2007).

1.8.2 Primary effusion lymphoma

Primary effusion lymphomas (PEL), also known as body cavity-based lymphomas, are a type of high grade non-Hodgkin's lymphoma that develop nearly exclusively in the pericardial, peritoneal and pleural cavities as lymphomatous effusion, usually in the absence of any identifiable tumour mass. Most cases occur in HIV-infected individuals (Cesarman and Knowles, 1997), but a small number of patients with PEL who are HIV-seronegative have been identified (Klepfish *et al.*, 2001). Cesarman and co-workers (1995a) reported that HHV-8 was specifically associated with PEL, and they noted that PEL contains high viral copies when grown *in vivo*, rendering them useful for the characterisation of individual strains of the virus. Phenotyping may be difficult because the cells characteristically lack the lineage-restricted markers of both T and B lymphocytes. Clonal immunoglobulin gene rearrangement found in all cases studied has established PEL as a B-cell neoplasm (Cesarman and Knowles, 1999; Carbone and Gloghini, 2005). Co-infection of PEL cells by both EBV and HHV-8 has been observed (Hamoudi *et al.*, 2004), but association with HHV-8 alone has also

been reported (Said *et al.*, 1996a; Cobo *et al.*, 1999). Notably, most PELs in HIV-seropositive patients are EBV-positive. However, EBV alone may not be sufficient for tumour development. Thus it is possible that HHV-8 acts in conjunction with EBV to induce full transformation (Miller, 1990; Cesarman *et al.*, 1995a). Thus far, no effective therapy has been established for HIV-associated PEL. Consequently, the prognosis is poor, and the reported median survival is about 18 weeks. Of interest are reports of clinical improvement after ART (Oksenhendler *et al.*, 1998b).

1.8.3 Multicentric Castleman's disease

Castleman's disease (CD) (angiofollicular lymphoid hyperplasia or giant lymph node hyperplasia) is a rare, non-malignant, usually polyclonal form of lymphadenopathy (Frizzera, 1992). It is classified histologically into the hyaline-vascular type and the plasma cell type. Clinically, it may be classified into two forms: (i) localized CD, which is usually of hyaline-vascular histologic type, is located in the mediastinum in 70% of affected patients, and can usually be cured by surgical excision; or (ii) a systemic variant, multicentric Castleman's disease (MCD), usually of plasma-cell type (Frizzera, 1992; Kardziev *et al.*, 2006). MCD adopts an aggressive, often fatal clinical course and usually presents with multifocal lymphadenopathy and a variety of systemic symptoms, such as fever, rash, cytopenia, and hypergammaglobulinemia. This disease is common among AIDS patients; HHV-8 DNA sequences could be found in all HIV-related cases of MCD, in contrast to less than half in HIV-seronegative cases (Soulier *et al.*, 1995). Moreover, HHV-8 viral load in the peripheral blood of MCD patients tends to correlate with the increased severity of symptoms, worse prognosis (Grandadam *et al.*, 1997) and exacerbations (Oksenhendler *et al.*, 2000). The clinical course of HHV-8-positive MCD tends to be poorer, particularly among HIV-seropositive individuals, and is not always improved by therapy with ART (Zietz *et al.*, 1999; Dupin *et al.*, 2000). HHV-8 DNA has been localised in plasmablasts of B cell lineage in the mantle zone of the B-cell follicles

(Kellam *et al.*, 1999; Katano *et al.*, 2000). The role of HHV-8 pathogenesis in MCD is not clear. However, vIL-6 is highly expressed in a subset of plasmablasts and may drive HHV-8-infected naïve B cells to become plasmablasts and to develop various lymphoproliferative lesions (Du *et al.*, 2001). In addition, patients with MCD may develop other HHV-8-associated diseases: PEL (Ascoli *et al.*, 2001), KS (Soulier *et al.*, 1995; Zietz *et al.*, 1999), or both (Codish *et al.*, 2000). Recently, a case of concomitant HHV-8-positive MCD and KS has been reported in the same lymph node of an HIV-negative liver transplant patient (Gaitonde *et al.*, 2007).

1.8.4 Other diseases

HHV-8 infection has been proposed as an aetiological agent in a number of other diseases including multiple myeloma (Said *et al.*, 1997; Hsu *et al.*, 2001; Csire *et al.*, 2007), some reactive lymphadenopathies (Luppi *et al.*, 1996), pemphigus vulgaris (Memar *et al.*, 1997; Jang *et al.*, 2000), a variety of skin carcinomas and lymphomas (Lazzi *et al.*, 2006), sarcoidosis (Di Alberti *et al.*, 1997b), primary pulmonary hypertension (Cool *et al.*, 2003), hemophagocytic syndrome (Re *et al.*, 2007), inflammatory myofibroblastic tumours (Gonzalez-Vela *et al.*, 2007) and Kikuchi disease (Cho *et al.*, 2007). Despite these associations, no conclusive evidence has been found to strongly link any of these diseases to infection with HHV-8.

1.9 HHV-8 epidemiology

1.9.1 Methods used to determine HHV-8 prevalence

HHV-8 DNA is readily detected by Southern blotting or PCR in KS, PEL and some lymphoid tissue from patients with MCD. Smaller amounts of viral DNA are present in non-neoplastic tissue from HHV-8-infected individuals, in particular in PBMCs, semen, urine, saliva and breast milk (Decker *et al.*, 1996; Blackbourn and Levy, 1997; LaDuca *et al.*, 1998; Dedicoat *et al.*, 2004; Beyari *et al.*, 2004). The detection rate of HHV-8 in PBMCs of individuals with KS is generally reported to be more than

50% and appears to be correlated with the stage and extent of KS (Campbell *et al.*, 2000; Camera *et al.*, 2000; Laney *et al.*, 2007). Likewise HHV-8 DNA detection and viral load in patients with active KS is more frequent than in patients with disease remission (Aluigi *et al.*, 1996; Albrecht *et al.*, 2004; Laney *et al.*, 2007). Furthermore, studies have suggested that the HHV-8 DNA detection rate in peripheral blood of KS patients and in patients subsequently progressing to KS is higher than in patients who do not develop KS or are at low risk of KS (Whitby *et al.*, 1995; Lefrere *et al.*, 1996; Moore *et al.*, 1996c; Smith *et al.*, 1997b; Min and Katzenstein, 1999; Engels *et al.*, 2003; Laney *et al.*, 2007). The reported HHV-8 genoprevalence in individuals without KS is variable, particularly in healthy individuals without HIV infection (ranging from 0% to 80%) (Dupon *et al.*, 1997; Kikuta *et al.*, 1997a; Cattani *et al.*, 1998; Belec *et al.*, 1998; Hudnall *et al.*, 2003; Zavos *et al.*, 2005).

Viraemia, measured as viral DNA in serum or plasma, has also been estimated (Bourboulia *et al.*, 2004). However, it has been reported that HHV-8 DNA in plasma in patients with KS may only be measured when the viral load in PBMCs is particularly high (Harrington *et al.*, 1996; Boivin *et al.*, 2002).

Many serological assay formats have been developed to test for the presence of HHV-8 antibodies and to complement testing by PCR. First generation assays were developed using HHV-8 infected PEL cell lines as the source of antigen. PEL cell lines express latent antigens, predominantly LANA which was the first serologic antigen identified by indirect immunofluorescence assay (IFA); while it is specific, it is generally less sensitive (about 80% sensitivity) (Gao *et al.*, 1996b). Treatment of PEL cells with TPA induces expression of lytic antigens. IFA using lytic antigens can yield higher sensitivity rates than LANA assays. However, they suffer from problems associated with cross reactivity to other herpesvirus antibodies (Lennette *et al.*, 1996). In an early IFA report, the cell line used (BC-1) was dually infected with HHV-8

and EBV, requiring absorption of EBV-specific antibodies to minimise cross-reactivity (Moore *et al.*, 1996b). Lytic replication of HHV-8, but not EBV, could be induced in BC-1 cell lines with n-butyrate, allowing the detection of antibodies to a prominent, 40-kDa, lytic structural antigen (Miller *et al.*, 1996). Although of limited use for determining the seroprevalence of HHV-8 in the general population, the results obtained with these early assays indicated that, not surprisingly, most individuals with AIDS-associated KS and a much smaller proportion of HIV-infected individuals without KS carried antibodies to HHV-8 (Miller *et al.*, 1996; Moore *et al.*, 1996c).

Other assays involve the detection of antibodies to unknown lytic antigens. These include an IFA using another PEL cell line, BCBL-1, treated with phorbol esters to induce the lytic replication cycle (Lennette *et al.*, 1996; Smith *et al.*, 1997b). With these assays, antibodies could be detected in nearly 100% of AIDS-associated KS patients and in 25% of United States blood donors (Lennette *et al.*, 1996).

Although assays using these lytic-phase PEL cell lines are the most sensitive and widely used, a dilemma is presented due to possible cross reactivity to other herpesvirus antibodies, particularly as no HHV-8 negative PEL cell lines exist to act as controls for non-specific reactions (Lennette *et al.*, 1996). This consideration has led to the development of second generation assays such as that described by Inoue *et al* (2000). In this IFA assay, recombinant ORF K8.1 antigen expressed by Semliki forest virus is used (Inoue *et al.*, 2000). This antigen has no homologue to other herpesviruses, hence cross-reactivity does not occur. The availability of negative controls lends specificity to this assay. An enzyme-linked immunosorbent assay (ELISA) based on a four-branch, multiple antigenic peptide derived from K8.1 has been shown to yield high sensitivity and specificity values when well-characterized HHV-8-infected individuals with KS were used as the positive controls and non-infected subjects as the negative controls (Lam *et al.*, 2002). However, in a recent

study, when non-KS individuals with high probability of HHV-8 infection were included in the positive control group, a significant reduction in the sensitivity was observed, implicating that inclusion of such a group may further improve a more accurate assessment of assay sensitivity (Perez *et al.*, 2006).

A purified whole-virion ELISA has been developed to facilitate high throughput testing. This rapid ELISA assay may be used to screen large numbers of sera to find those at risk for developing KS (Chatlynne *et al.*, 1998). To further increase sensitivity and specificity, other ELISAs have been developed using, as antigens, various recombinant proteins and oligopeptides (Andre *et al.*, 1997; Davis *et al.*, 1997a; Pau *et al.*, 1998). Antigens commonly utilised in this type of serological assay include products from ORF 65 (Pau *et al.*, 1998), ORF 73 (Gao *et al.*, 1999) and ORF K8.1 (Perez *et al.*, 2006). However, these peptide ELISAs can be used for trends in populations with high probability of being HHV-8 infected, and negative results should be confirmed by IFA (Perez *et al.*, 2006).

A number of studies comparing the variable performance of different serological HHV-8 tests exist (Rabkin *et al.*, 1998; Spira *et al.*, 2000; Schatz *et al.*, 2001; Pellett *et al.*, 2003; Sergerie *et al.*, 2004). Evidence suggests that patients show heterogeneous serologic responses to defined HHV-8 antigens; therefore, combinations of antigen assays may achieve the best detection rates (Lang *et al.*, 1999; Spira *et al.*, 2000; Laney *et al.*, 2006; Bergallo *et al.*, 2007) and the use of a combination of both latent and lytic assays to evaluate seropositivity should avoid the possibility of false negative results and provide an exact evaluation of the rate of seroconversion (Marcelin *et al.*, 2007). Recently, a combined quantitation of both humoral and cellular immunity has been suggested to be a superior method in assigning HHV-8 infection status (Lonard *et al.*, 2007).

Although some studies suggest wide differences in infection rates in populations at risk of KS based on the type of antigen and serological method used (Rabkin *et al.*, 1998; Enbom *et al.*, 2000; Sergerie *et al.*, 2004), properly performed assays in standardized formats have been found to be highly concordant (Schatz *et al.*, 2001).

1.9.2 HHV-8 seroprevalence and geographical distribution

Many studies have been undertaken to determine the seroprevalence of antibodies to HHV-8. The seroepidemiologic studies clearly show that HHV-8 is prevalent throughout the world, although there may be local variations in seroprevalences (Whitby *et al.*, 1995; Gao *et al.*, 1996b; Kasolo *et al.*, 1997; Whitby *et al.*, 1998; Rezza *et al.*, 1998; Olsen *et al.*, 1998; Perna *et al.*, 2000; Iscovich *et al.*, 2000; Chokunonga *et al.*, 2000; Schulz, 2000a; He *et al.*, 2007). A variety of techniques have been used in these studies, but to date there is no gold standard against which to measure the efficacy of these techniques, so some may overestimate and others underestimate the true prevalence (Laney *et al.*, 2006; Perez *et al.*, 2006).

Unlike most human herpesviruses, HHV-8 is not spread universally among all human populations. The highest seroprevalences in healthy populations are found in sub-Saharan Africa (Ablashi *et al.*, 1999; de The *et al.*, 1999; Schulz, 2000b), where the prevalence among adults ranges from 22% in central Africa to 71% in Zambia (Olsen *et al.*, 1998; Belec *et al.*, 1998; He *et al.*, 1998). Very high rates, between 79% and 87%, have been reported in the Congo and in Botswana (Engels *et al.*, 2000). In black South African blood donors and patients with cancer other than KS, the seroprevalence is 32% (Sitas *et al.*, 1999a). In Mediterranean countries, the seroprevalence averages 8% to 10%, although population pockets where the prevalence is as high as 20% can be found (Angeloni *et al.*, 1998; Whitby *et al.*, 1998; Calabro *et al.*, 1998; Chatlynne and Ablashi, 1999; Schulz, 2000b; Davidovici *et al.*, 2001; Vitale *et al.*, 2001; Zavitsanou *et al.*, 2007). In three studies undertaken

in Saudi Arabia, serum antibodies to HHV-8 were detected in 7%, 3.9% and 1.7% of healthy participants (Qunibi *et al.*, 1998; Almuneef *et al.*, 2001; Alzahrani *et al.*, 2005). In the United States, seroprevalences ranging from 0% to 26% have been reported (Lennette *et al.*, 1996; Gao *et al.*, 1996b; Baillargeon *et al.*, 2002; Pellett *et al.*, 2003; Martro *et al.*, 2004; Casper *et al.*, 2006). In northern Europe, Germany, Cuba, Southeast Asia, and the Caribbean, seroprevalence falls into the 1% to 4% range (Ablashi *et al.*, 1999; Kouri *et al.*, 2004; Zavitsanou *et al.*, 2007; Lonard *et al.*, 2007) with the seroprevalence of HHV-8 antibodies in blood donors from Japan estimated as 0.2% to 3.2% (Fujii *et al.*, 1999; Satoh *et al.*, 2001). However, some surveys found the antibody prevalence to HHV-8 to be 19% in Taiwan (Huang *et al.*, 2000), 47% in northwest China (Dilnur *et al.*, 2001) and 20% in Sweden (Enbom *et al.*, 2000); the later report also showing a wide variety of results depending on the assay used.

HHV-8 seroprevalences in South America have also been documented. The seroprevalence in blood donors was found to be 4% from Argentina and 3% from Chile (Perez *et al.*, 2004). In Brazil, varying seroprevalences - upto 79% among Amerindians (Biggar *et al.*, 2000; Ishak *et al.*, 2007; de Souza *et al.*, 2007b), 16% in urban communities (Freitas *et al.*, 2002), 6% in non-Amerindian populations living on the banks of the Trombetas river (de Souza *et al.*, 2007b) and 2.8% in Campinas and surrounding cities (Perez *et al.*, 2004) - have been reported. In Peruvian blood donors, HHV-8 seroprevalence was recently found to be as high as 56% (Mohanna *et al.*, 2007).

In many populations, especially those in northern Europe, Brazil and North America, the HHV-8 seroprevalence is 2 to 10 times higher in men than women (Whitby *et al.*, 1995; Cesarman *et al.*, 1995b; Lennette *et al.*, 1996; Kedes *et al.*, 1997; Chatlynne and Ablashi, 1999; Greenblatt *et al.*, 2001; Perez *et al.*, 2004). Some of these

differences may be due to higher seroprevalence in homosexual than heterosexual men and women. However, a recent study has reported a significantly higher seroprevalence amongst Brazilian Amerindian females than males (Ishak *et al.*, 2007). In other populations such as Israel (Iscovich *et al.*, 2000), Greece (Zavitsanou *et al.*, 2007), Argenitina and Chile (Perez *et al.*, 2004) the seroprevalence was found to be similar in men and women. While a complete picture of the seroepidemiology of HHV-8 in the African continent is not yet available, all surveys to date indicate that HHV-8 antibodies are seen in about equal frequency in men and women (Olsen *et al.*, 1998; Gessain *et al.*, 1999).

The seroprevalence in children tends to reflect that of the adult population, but the percentages in children are lower than that of the adults. Within Africa, the HHV-8 seroprevalence among children ranges from 13% in Cameroon (Gessain *et al.*, 1999) to 37% in Uganda (Mayama *et al.*, 1998), and from 36% in Ghana (Nuvor *et al.*, 2001) to 47% in Zambia (Olsen *et al.*, 1998). An HHV-8 prevalence of 4% has been reported among Italian children younger than 11yr (Whitby *et al.*, 2000). The highest prevalence in the Mediterranean area, of up to 45%, has been reported among children in Alexandria, Egypt (Andreoni *et al.*, 1999). In the USA, Baillargeon *et al.* (2002) reported the overall prevalence of HHV-8 in South Texas children to be 26% - a rate higher than that reported in other studies of children in North America and Europe (Blauvelt *et al.*, 1997; Whitby *et al.*, 2000; Martro *et al.*, 2004).

Although US children aged 6 months to 5 yr tend to have higher HHV-8 antibody titres than 6 -17 yr old children (Martro *et al.*, 2004), most studies describe an increase in seroprevalence with age amongst other populations (Rezza *et al.*, 1998; Sitas *et al.*, 1999a; Whitby *et al.*, 2000; Almuneef *et al.*, 2001; Stein *et al.*, 2004; Zavitsanou *et al.*, 2007; de Souza *et al.*, 2007b). In African countries with high seroprevalence, such as Cameroon (Gessain *et al.*, 1997), French Guiana

(Plancoulaine *et al.*, 2000), Nigeria (Martro *et al.*, 2004) and Uganda (Mayama *et al.*, 1998), seropositivity increases with age and usually reaches the prevalence of adult groups before puberty.

HIV-infected homosexual men have the highest HHV-8 seroprevalence of any risk group. A study in the San Francisco area, using serum samples dating to 1984, reported a seroprevalence of 48% among homosexual or bisexual men who were HIV-positive but did not have KS (Martin *et al.*, 1998). Another study from the US found the seroprevelance of HHV-8 in homosexual and heterosexual United States military men with HIV infection to be 35% and 27% respectively (Crum *et al.*, 2003). In Europe and North America, HIV-1-seronegative homosexual men consistently show a lower seroprevalence of HHV-8 infection than HIV-1-seropositive homosexual men, and higher seroprevalence than observed among blood donors or the general population (Kedes *et al.*, 1996; Melbye *et al.*, 1998; Martin *et al.*, 1998; Casper *et al.*, 2006; Engels *et al.*, 2007).

In recent studies of HIV-infected patients without KS, the HHV-8 seroprevalence was found to be 19% in Hungary (Szalai *et al.*, 2005), 28% in Northern Thailand (Chen *et al.*, 2004), 30% in Amsterdam (Dukers *et al.*, 2000), 45% in Eastern Sicily (Larocca *et al.*, 2005), and up to 52% in Germany (Albrecht *et al.*, 2004; Lonard *et al.*, 2007). Antibody titres against HHV-8 LANA, assessed by IFA, were found to positively correlate with the CD4+ T-cell count of HIV-positive patients with KS, a finding important for the interpretation of seroepidemiological studies of HHV-8 infection in AIDS patients (de Souza *et al.*, 2007a). While HHV-8 seropositivity correlated with high CD4+ T-cell counts (de Souza *et al.*, 2007a), clinically manifest KS was more likely in patients with low than high CD4+ T-cell counts (Martro *et al.*, 2007).

The results of serologic studies support the view that infection with HHV-8 is nearly

universal in patients with KS. For example, 100% of Moroccan patients with classical or endemic KS (Hbid *et al.*, 2005), 100% of Tanzanian patients with endemic or AIDS-associated oral KS (Mwakigonja *et al.*, 2007), 83% to 100% of patients with African endemic KS (Lennette *et al.*, 1996; Sitas *et al.*, 1999a), and 96% of U.S patients with AIDS-associated KS (Lennette *et al.*, 1996) were found to be seropositive for antibodies against lytic antigens. The geographic association between KS prevalence and HHV-8 seroprevalence is significant. Populations in Southern Italy, Sardinia and Sicily, where KS rates are high, show HHV-8 seroprevalences between 25-30% (compared with 10% in Northern Italy), and in the rest of Europe and North America the seroprevalence ranged between 1-8% (Whitby *et al.*, 1995; Angeloni *et al.*, 1998; Antman and Chang, 2000). The seroprevalence in Africa also appears to mirror the epidemiology of KS, although high HHV-8 antibody titres have been suggested to protect against circulating HHV-8 in PBMC or oral fluids and new KS lesions (Cannon *et al.*, 2003).

HHV-8 seroconversion precedes KS in all populations studied (Gao *et al.*, 1996a; Renwick *et al.*, 1998; Oksenhendler *et al.*, 1998a). However, several factors may influence the risk of developing KS in patients with prior HHV-8 infection, including the ethnic origin of the patients, the seroprevalence rate in their geographic origin, gender, age, and degree of immunosuppression (Lennette *et al.*, 1996; Gao *et al.*, 1996b; Penn, 1997; Calabro *et al.*, 1998). A continued state of immunodeficiency is important for the development of KS (Zavos *et al.*, 2004).

1.9.3 Comparison of HHV-8 seroprevalence and genoprevalence

The absence of gold standard serological tests and sensitive methods for detecting low copy numbers of the HHV-8 genome in PBMCs has contributed to the uncertainty over the true prevalence of this virus in the general population of non-KS-endemic regions. However, many groups have attempted to improve sensitivity in detecting

low copy numbers of viral DNA by employing nested PCR, and a wide range of approaches have been employed (Cattani *et al.*, 1998; Belec *et al.*, 1998; Min and Katzenstein, 1999; Spira *et al.*, 2000). The use of these improved methods to determine HHV-8 genoprevalence may better mirror the true prevalence in these individuals. Furthermore, a comparison of geno- and sero-prevalence may assist in clarifying the specificity and sensitivity of serological methods. Such data may also allow for assessment of the stage of HHV-8 infection: primary, latent, or lytic. In general, people who are HHV-8 seropositive without detectable DNA in the peripheral blood may be considered to be latently infected (Simpson *et al.*, 1996; Gao *et al.*, 1996a), seronegative people who are only DNA-positive to be hosting an early primary infection (Andreoni *et al.*, 2002), and those who are positive in both tests to be undergoing reactivated infection or late primary infection.

1.10 Organ transplantation and human herpesvirus infections

Viral infections among renal transplant recipients continue to be a major cause of post-transplantation morbidity and mortality despite the availability of effective antiviral therapy (Briggs, 2001; Hwang *et al.*, 2004). Human herpesviruses are found worldwide, being among the most frequent causes of viral infections in immunocompetent as well as immunocompromised patients. In transplant recipients, herpesviruses may be acquired via the transplanted organ or blood products or may be reactivated as a consequence of the use of powerful immunosuppressive drugs (Walker *et al.*, 1982; Smith *et al.*, 2001). Moreover, the persistence of immunosuppression greatly favours the clinical expression and severity of virus infection. Thus, herpes viruses are frequently involved in both acute and chronic deterioration of graft function, in the pathogenesis of post-transplant lymphoproliferative disorders and KS, and even in cardiovascular events (Brennan, 2001; Morrone *et al.*, 2002; Gomez *et al.*, 2005).

HSV infection may occur in renal allograft recipients mainly during or shortly after treatment of graft rejection with high-dose steroids (Mosimann *et al.*, 1994; Kang *et al.*, 2006). Herpes simplex infections present as mucocutaneous lesions that may persist for months with dissemination to internal sites, including the esophagus, colon, and bladder. The infection usually responds to aciclovir (Hwang *et al.*, 2004; Kang *et al.*, 2006).

Chickenpox caused by VZV infection is a rare infection in adult renal transplant recipients; however, it is significant owing to the severity of its clinical features and its associated high mortality rate. All renal transplant recipients should be screened for VZV immunization prior to transplant irrespective of previous history of VZV infection (Lauzurica *et al.*, 2003; Shahbazian and Ehsanpour, 2007). Renal transplant recipients with chickenpox can present with atypical symptoms such as acute abdominal pain and low back pain (Shahbazian and Ehsanpour, 2007). Early diagnosis and treatment with high doses of aciclovir is fundamental in infection control (Lauzurica *et al.*, 2003). Vaccination should preferably be administered in seronegative recipients several months prior to transplant (Geel *et al.*, 2006; Robertson *et al.*, 2006; Shahbazian and Ehsanpour, 2007).

Primary EBV infection is the most important risk factor for development of post-transplant lymphoproliferative disorder (PTLD) (Smith *et al.*, 2007). Transmission of EBV can occur via an allograft with subsequent infection of a previously seronegative recipient (Cen *et al.*, 1991; Haque *et al.*, 1996). The PTLD presentation is variable. Some patients present asymptomatically; in others, early symptoms can be nonspecific. Screening for patients at risk, balancing the intensity of immunosuppressive regimens against the risk of rejection and using antiviral agents active against EBV can substantially reduce the risk of developing PTLD. Depending on the severity of PTLD, treatment may necessitate reduction of immunosuppression

with or without radiotherapy, interferon-alpha, or additional chemotherapy (Everly *et al.*, 2007).

CMV is a significant post-transplant pathogen and continues to be a common cause of morbidity and mortality in transplant recipients. The wide incidence of reported CMV infection and disease (Hwang *et al.*, 2004) probably reflects differences in immunosuppressive strategies and monitoring protocols for such disease. CMV infection can initiate endothelial cell activation and vascular injury that may facilitate acute or chronic graft rejection, atherosclerosis, transplant glomerulopathy or thrombotic microangiopathy (Brennan, 2001). The clinical manifestations of CMV disease in transplant recipients range from asymptomatic infection to mild mononucleosis syndromes to severe multisystem involvement (e.g. retinitis, pneumonitis and gastrointestinal disease). Several studies have shown that CMV-seronegative recipients of an organ from a seropositive donor are at much greater risk of acquiring a primary infection and it is generally accepted that primary infections are more severe than reactivated infections (Peterson *et al.*, 1980; Pollard, 1988). Ganciclovir and valganciclovir are effective in the treatment of CMV disease (Fellay *et al.*, 2005; Luan *et al.*, 2006) and for CMV prevention (Paya *et al.*, 2004) in high-risk solid organ transplant recipients. Pre-emptive valganciclovir therapy and prophylaxis using valganciclovir (Khoury *et al.*, 2006) or valaciclovir (Reischig *et al.*, 2007) have been found to be equally effective in the prevention of CMV disease after renal transplantation.

HHV-6 has been described as an immunomodulatory and immunosuppressive virus that may facilitate superinfections with other opportunistic organisms in transplant recipients, particularly CMV (Flamand *et al.*, 1995; Dockrell *et al.*, 1997; Singh, 2000). HHV-7 also appears to be an immunomodulatory agent and may facilitate the pathogenicity of cytomegalovirus. In a study of renal transplant recipients, patients

with CMV and HHV-7 coinfection were more likely to have CMV disease than those with CMV infection alone (Kidd *et al.*, 2000).

HHV-8 may be transmitted via the transplanted organ (Parravicini *et al.*, 1997; Luppi *et al.*, 2000a; Kapelushnik *et al.*, 2001) or may be reactivated in patients who were infected before allograft receipt (Cattani *et al.*, 2001; Bergallo *et al.*, 2007). The most commonly reported clinical manifestation of HHV-8 infection among transplant recipients is the development of KS.

1.11 Renal transplantation and KS

Iatrogenically immunosuppressed individuals, including renal allograft recipients, are at risk of developing malignant tumours caused by viral infections (Penn, 1983; Farge, 1993). KS was first reported in renal transplant recipients in 1969 and since then has been described with increasing frequency in recipients of kidney and other solid organ transplants given conventional immunosuppressive therapy (azathioprine plus prednisolone) or ciclosporin (Farge, 1993). The prevalence of KS among European transplant recipients is about 0.5% and appears to be slightly higher in liver (1.2%) compared with kidney (0.5%) and heart (0.4%) transplant recipients (Farge, 1993).

The incidence of KS in renal transplant recipients is 84 to 500-fold greater than in control populations (Harwood *et al.*, 1979; Montagnino *et al.*, 1994; Montagnino *et al.*, 1996; Jensen *et al.*, 1999; Saadat *et al.*, 2007) and varies from less than 0.01% to 5.3% (Harwood *et al.*, 1979; Qunibi *et al.*, 1988; Shmueli *et al.*, 1989; Qunibi *et al.*, 1993; Farge, 1993; al Sulaiman and al Khader, 1994; Penn, 1995; Cathomas *et al.*, 1997; Mbulaiteye and Engels, 2006; Garcia-Astudillo and Leyva-Cobian, 2006; Bergallo *et al.*, 2007; Vegso *et al.*, 2007). Such variation is influenced by whether the allograft recipients and donors originate from regions where KS is endemic

(Franceschi and Geddes, 1995), and by the type of regimen used for post-transplantation immunosuppression (Farge, 1993; Penn, 1996). Amongst allograft recipients, KS has been reported to affect men more than women in a 3:1 ratio (Penn, 2000), a figure far less than the 9:1 to 15:1 ratio seen with KS in the general population (Penn, 1989; Penn, 1994; Penn, 1995).

In Saudi Arabia, the incidence of KS among renal transplant recipients is more than 10 times higher than among recipients in the United States and western Europe (Farge, 1993; Mbulaiteye and Engels, 2006) and accounts for over 70% of all neoplasms experienced by renal transplant recipients (Qunibi *et al.*, 1988; Qunibi *et al.*, 1993; al Sulaiman and al Khader, 1994) compared to < 0.4% (al Suleiman *et al.*, 1987; Qunibi *et al.*, 1988) of all malignancies in Saudi Arabia. KS is the most common post-transplant tumour in Saudi Arabian (Qunibi *et al.*, 1988; Qunibi *et al.*, 1993; al Sulaiman and al Khader, 1994), Pakistani (Askari *et al.*, 1999), Turkish (Ecder *et al.*, 1998; Duman *et al.*, 2002) and Egyptian (El Agroudy *et al.*, 2003) renal allograft recipients. The mean period between transplantation and diagnosis of KS has been reported to be 12.5 months (Qunibi *et al.*, 1988), 13.6 months (al Suleiman *et al.*, 1987) and 15.6 months (Qunibi *et al.*, 1993) in Saudi Arabia, 15.9 months to 24 months in Turkey (Ecder *et al.*, 1998; Moray *et al.*, 2004), 17.1 months in Pakistan (Askari *et al.*, 1999), 19.6 months in Hungary (Vegso *et al.*, 2007) and 33.8 months in Spain (Garcia-Astudillo and Leyva-Cobian, 2006). The median duration between transplantation and KS onset has been reported to be 7 months in Italy (Parravicini *et al.*, 1997).

HHV-8 DNA has been detected in peripheral blood of allograft recipients (Aluigi *et al.*, 1996; Kikuta *et al.*, 1997a; Zhu *et al.*, 2008) and the HHV-8 seroprevalence in renal transplant recipients has been found to be 50% in the USA (Hudnall *et al.*, 1998), 26% in Italy (Cattani *et al.*, 2001), 41% in China (Zhu *et al.*, 2008), 25% in Iran

(Ahmadpoor *et al.*, 2007), 22% in Germany (Lonard *et al.*, 2007), 0.6% in Spain (Garcia-Astudillo and Leyva-Cobian, 2006) and zero in Cuba (Kouri *et al.*, 2004).

Although haemodialysis patients have been found to be more susceptible to viruses, such as HHV-8, and hence in danger of developing KS (Hsu *et al.*, 2002), the connection between dialysis, HHV-8, and KS is not yet well established (Herr *et al.*, 2001). However, KS is rarely found in haemodialysis patients and only a few cases have been reported to occur as early as with the onset of dialysis (Herr *et al.*, 2001; Metaxa-Mariatou *et al.*, 2004; Yasar *et al.*, 2007). In Switzerland - a well known low-risk population - a HHV-8 seroprevalence of 6.4% was found among patients with renal failure before transplantation (Regamey *et al.*, 1998). In Italy, Cattani *et al.*, 2001, found no significant difference in the HHV-8 seroprevalence between pre-transplant patients and appropriate control subjects (Cattani *et al.*, 2001), while Bergallo *et al.* (2007) observed a higher HHV-8 seroprevalence rate in end-stage renal disease (ESRD) patients when compared to their corresponding allograft donors. Earlier studies reported a higher pre-transplantation seroprevalence rate among Italian females (Andreoni *et al.*, 2001), while later studies found no difference in prevalence by gender (Bergallo *et al.*, 2007), both not consistent with the higher KS incidence previously found among males in Italy (Cottoni *et al.*, 1996). An association between HHV-8 seroprevalence and increasing age in ESRD patients has been reported from Saudi Arabia (Almuneef *et al.*, 2001) and Italy (Bergallo *et al.*, 2007).

In Saudi Arabia, the HHV-8 seroprevalence in renal transplant recipients is high when compared to other countries in which such estimates have been made. In a small seroprevalence study of Saudi Arabian patients, the seropositivity rate was 93% in patients with post-transplantation KS, 28% in transplant recipients without KS, and 29% in patients with end-stage renal disease (ESRD) on haemodialysis (Qunibi *et al.*, 1998). In a larger study of individuals in Saudi Arabia, the same researchers found

antibodies to HHV-8 in only 7% of ESRD patients (Almuneef *et al.*, 2001), this being relatively similar to that detected recently in haemodialysis patients in Greece (7.2%) (Zavitsanou *et al.*, 2006) but less than that of haemodialysis patients in Germany (13.7%) (Lonard *et al.*, 2007). More recently, in Saudi Arabia, 18% of renal transplant recipients and seven out of 10 (70%) family members of a renal allograft recipient with KS were found to be seropositive for HHV-8 (Alzahrani *et al.*, 2005). Seropositivity for HHV-8 in these transplant patients was not significantly influenced by the existence of relatives with kidney failure, the donor's country of origin, the recipient's home region within Saudi Arabia, the haemodialysis centre, the time that elapsed since the renal transplantation operation, and the immunosuppressive regimen employed (Almuneef *et al.*, 2001; Alzahrani *et al.*, 2005).

Among renal transplant recipients in Saudi Arabia, the skin is the most common organ involved with KS, followed by the gastrointestinal tract and the lungs (Qunibi *et al.*, 1993; al Sulaiman and al Khader, 1994). Oral KS has also been observed in this group of patients (Qunibi *et al.*, 1988; Qunibi *et al.*, 1993). The prognosis in patients with KS limited to the skin is favourable (probably because of early detection and treatment), while visceral involvement is associated with high mortality (al Sulaiman and al Khader, 1994; Zavos *et al.*, 2004; Veroux *et al.*, 2004). Patients with iatrogenic KS benefit from reduction or cessation of immunosuppression, but there is a high risk of graft loss (Veroux *et al.*, 2004; Moray *et al.*, 2004).

Post-transplantion KS may be caused by two possible mechanisms: HHV-8 reactivation in patients who were infected before the graft and HHV-8 neo-infection either by blood products or from allogeneic transplant tissue (Marcelin *et al.*, 2007). However, most cases of post-transplant KS apparently develop as a result of viral reactivation. Among patients who are seropositive for HHV-8 before undergoing renal transplantation, the risk of post transplant KS is 23% to 28%, as compared with a risk

of 0.7% in patients who are seronegative before receiving a renal transplant (Frances *et al.*, 2000; Cattani *et al.*, 2001), suggesting that infection prior to transplantation is a key risk factor for KS (Cattani *et al.*, 2001). However, not all HHV-8 seropositive patients before renal transplant and undergoing immunosuppression develop KS (Parravicini *et al.*, 1997; Frances *et al.*, 2000; Cattani *et al.*, 2001; Alzahrani *et al.*, 2005; Bergallo *et al.*, 2007) and the incidence of KS among HHV-8 seropositive patients may in some areas be very low (Bergallo *et al.*, 2007).

The degree of immunosuppression is a critical factor in the likely clinical development of iatrogenic KS in renal allograft recipients. It has been demonstrated that renal transplant recipients have a higher incidence of KS than non-grafted patients on maintenance dialysis (Montagnino *et al.*, 1996; Qunibi *et al.*, 1998). While some *in vitro* studies have shown that immunosuppressive agents such as hydrocortisone and ciclosporin do not activate the lytic cycle of HHV-8 and do not modify the cell survival (Marcelin *et al.*, 2001), others have found ciclosporin to have a tumour promoting activity (Guba *et al.*, 2002) and hydrocortisone able to activate the lytic cycle of HHV-8 (Hudnall *et al.*, 1999). Administration of more than 10 corticosteroid pulses has been found to be associated with an increased risk of disease (Montagnino *et al.*, 1996). However, it still remains unclear as to how the use of these different immunosuppressive agents influences the development of KS in renal allograft recipients (Cockburn and Krupp, 1989; Hiesse *et al.*, 1995; Rezeig *et al.*, 1997; Farge *et al.*, 1999; Eberhard *et al.*, 1999).

1.12 Importance of research reported in this thesis

Despite recent advances in transplantation techniques, herpesvirus infections remain a major cause of morbidity and mortality in transplant recipients. Improvements in immunosuppressive drug regimens have decreased the risk of rejection in solid organ transplant recipients. However, therapy with all such agents carries an increased risk

of herpesvirus reactivation. The most commonly reported clinical manifestation of HHV-8 infection among transplant recipients is the development of KS. The rate of KS among solid-organ transplant recipients varies by geographic location, such that countries with endemic KS and higher rates of seropositivity to HHV-8 seem to have higher rates of KS among the transplant population. However, this situation may not apply to Saudi Arabia.

This study was prompted by the following observations:

1. The reported disparity in HHV-8 prevalence rates between the general population and patients with renal disease in Saudi Arabia. This phenomenon has not been reported elsewhere in the world, and the reason for the particularly high incidence of post-transplantation KS in Saudi Arabia is unknown.

2. Previous epidemiological studies in the Saudi population have relied greatly on serological data to estimate the prevalence of HHV-8 infection. In view of the uncertain specificity and sensitivity of serological estimations of HHV-8 seroprevalence, it was considered necessary to test samples for both geno- and sero-prevalence to ensure that HHV-8 infection, regardless of its stage, does not go undetected. Genoprevalence studies potentially enhance the specificity of, and lend sensitivity to, estimations of HHV-8 prevalence in populations at low risk of KS.

3. No studies into the oral shedding of HHV-8 in the Saudi Arabian context, particularly involving patients with renal disease, who are at particular risk of KS exist. In other geographical populations and other subpopluations that are at high risk of HHV-8 infections, there is gathering consensus that HHV-8 is shed predominantly from the mouth.

1.13 Hypothesis

There is evidence that transplant-associated KS is more prevalent among renal allograft recipients in Saudi Arabia than some other geographic regions of the world. Given the known association between HHV-8 infection and risk of developing KS, it was hypothesised that the increased frequency of KS in renal allograft recipients results from HHV-8 infection prior to transplantation and reactivation of HHV-8 post transplantation when the patients are given intensive immunosuppression.

1.14 Study Aims

The study has been undertaken in an attempt to evaluate the extent of HHV-8 carriage in saliva, oral mucosal surfaces and peripheral leukocyte subsets and to explore the factors that may influence the shedding of the virus in the study groups representing populations with different risks of HHV-8 infection, including ESRD patients and patients who have undergone renal transplantation. The genetic variation of the K1 gene of HHV-8 was analysed and the possibility of multiple infection examined.

Chapter 2

Patients, Materials and Methods

2.1 Patients and samples

Samples were collected from eight different groups of participants attending the Armed Forces Hospital in Riyadh, Saudi Arabia. These eight groups included: blood donors (hereafter designated the "BD" group) (n=178), pregnant women visiting the prenatal clinic (the "PW" group) (n=60), patients with chronic renal failure undergoing haemodialysis (the "CRF" group) (n=72), renal allograft recipients with no history of KS ("RAR") (n=61), renal allograft recipients with a history of KS ("RAR-KS") (n=5), patients with chronic renal failure undergoing kidney transplant ("CRF-tr") (n=6), renal allograft donors ("RAD") (n=6), and relatives of renal allograft recipients ("RRAR") (n=3). As the number of females donating blood at the designated study centre was found to be low, the inclusion of pregnant women attending the prenatal clinic was found to be necessary. The minimum number of participants in each of the four major study groups (BD, PW, CRF and RAR groups), required to detect a significant difference, was determined on the basis of previously published relevant data, the selected statistical power (80%) and *P* value (≤ 0.05). Ethical approval for the study was obtained from the Ethical Committee of the Riyadh Armed Forces Hospital.

The clinical, virological and immunological characteristics determined for each of the eight groups are listed in Table 2.1. Oral findings and indices (plaque, gingival and bleeding indices) were documented using a standard proforma (Appendix 1).

Table 2.1 Clinical, virological and immunological characteristics obtained for examined patient groups

Group	Clinical	Virological	Immunological
BD	▪ Patient age and gender ▪ ABO blood group	▪ PBMC HHV-8 DNA	▪ Serological evidence of HIV, HBV and HCV infection ▪ Anti-HHV-8 IgG
PW	▪ Patient age and week of pregnancy	▪ PBMC HHV-8 DNA	▪ Serological evidence of HBV or HCV infection ▪ Anti-HHV-8 IgG
CRF	▪ Patient age, gender and area of residence (past and current) ▪ Dialysis shift and assigned unit location ▪ Medical history, medications, previous transplants and duration of dialysis ▪ Systematic examination for oral lesions and documentation ▪ Oral hygiene status and presence or absence of gingival disease	▪ PBMC HHV-8 DNA ▪ Whole mouth and parotid saliva HHV-8 DNA ▪ Buccal and palatal HHV-8 DNA	▪ Serological evidence of HIV, HBV and HCV infection ▪ Anti-HHV-8 IgG
RAR & RAR-KS	▪ Patient age, gender and area of residence (past and current) ▪ Medical history and medications ▪ Date of allograft receipt and type of allograft (live or cadaver donor) ▪ Location of transplantation and nationality of donor ▪ Systematic examination for oral lesions and documentation ▪ Oral hygiene status and presence or absence of gingival disease ▪ Immunosuppressive drug regimen and administered dose ▪ Presence or absence of KS or other known malignancies	▪ PBMC HHV-8 DNA ▪ Plasma HHV-8 DNA ▪ Whole mouth and parotid saliva HHV-8 DNA ▪ Buccal and palatal HHV-8 DNA ▪ Paraffin embedded KS biopsy HHV-8 DNA	▪ Serological evidence of HBV or HCV infection ▪ Anti-HHV-8 IgG

BD, blood donors; PW, pregnant women; CRF, patients with chronic renal failure; RAR, renal allograft recipients with no history of KS; RAR-KS, renal allograft recipients with a history of KS

Table 2.1 Clinical, virological and immunological characteristics obtained for examined patient groups ...*continued*

Group	Clinical	Virological	Immunological
CRF-tr	▪ Patient age, gender and area of residence (past and current) ▪ Medical history and medications ▪ Type of allograft (live or cadaver donor) ▪ Nationality and relation of donor ▪ Immunosuppressive drug regimen, dose and duration* ▪ Systematic examination for oral lesions and documentation* ▪ Oral hygiene status and presence or absence of gingival disease* ▪ Presence or absence of KS or other known malignancies*	▪ PBMC HHV-8 DNA* ▪ Plasma HHV-8 DNA* ▪ Whole mouth and parotid saliva HHV-8 DNA* ▪ Buccal and palatal HHV-8 DNA *	▪ Serological evidence of HIV, HBV and HCV infection* ▪ Anti-HHV-8 IgG*
RAD	▪ Patient age, gender and area of residence (past and current) ▪ Medical history and medications	▪ PBMC HHV-8 DNA ▪ Plasma HHV-8 DNA ▪ Whole and parotid saliva HHV-8 DNA ▪ Buccal and palatal HHV-8 DNA	▪ Serological evidence of HIV, HBV and HCV infection ▪ Anti-HHV-8 IgG
RRAR	▪ Patient age and gender	▪ PBMC HHV-8 DNA ▪ Plasma HHV-8 DNA	▪ Serological evidence of HIV, HBV and HCV infection ▪ Anti-HHV-8 IgG

CRF-tr, patients with chronic renal failure undergoing kidney transplant; RAD, renal allograft donors; RRAR, relatives of renal allograft recipients
*Undertaken prior to, and at weeks 1, 2, 4, 6 and 8, and 9 months after renal transplant

2.2 Sample Preparation

2.2.1 Whole blood

Peripheral blood from study individuals was collected in EDTA-treated vacutainers by venepuncture, and stored, if necessary, for not more than 24 h at 4°C. Blood collection was carried out by assigned nurses in the presence of the researcher. Plasma was separated from blood by centrifugation at 3500 rpm for 15 min with 1 ml of plasma removed and stored at -20°C for later serological studies. The remaining blood was resuspended and immunomagnetic cell separation was achieved using Dynabeads (Dynal A.D., Oslo, Norway).

Immunomagnetic cell isolation using Dynabeads allows isolation of predefined subsets directly from heterogeneous cell suspensions, such as whole blood. Dynabeads (Dynal A.D., Oslo, Norway) are small magnetically charged uniformly shaped beads coated with a specific monoclonal antibody. The Dynabeads are supplied as a suspension containing $4x10^8$ beads/ml in phosphate buffer saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide (NaN_3). Because NaN_3 is a cytotoxic agent, the beads require to be washed before use with a washing buffer (PBS/0.1% BSA).

2.2.1.1 Dynabeads washing procedure

The desired amount of Dynabeads was transferred into a washing tube. The tube was then placed on a magnetic particle concentrator (Dynal MPC) for 60 s and the fluid was pipetted off carefully. After the tube was removed from the magnetic device, 1 ml PBS/0.1% BSA was added and the beads resuspended. This procedure was repeated another two times before the Dynabeads were resuspended back into their original volume.

2.2.1.2 Immunomagnetic cell isolation

To separate the different leukocyte fractions from the blood, Dynabeads coated with antibodies against CD45+, CD31+, CD19+, CD14+ and CD2+ cell subsets were added directly to the cell suspension, where they bind the target cells. The cell types targeted in the current investigation and their cell surface cluster of differentiation (CD) markers are summarised in Table 2.2.

To 1 ml of whole blood, 50 µl of Dynabeads were added giving an approximate final concentration of $2x10^7$ beads/ml. The whole blood and Dynabead mixture was then incubated at 4°C for 1 h providing rotation of the tube every 10 min using a Dynal sample mixer (Dynal A.D., Oslo, Norway). The mixture tube was then positioned in the magnetic particle concentrator (Dynal MPC) for 3 min to collect the rosetted cells. The supernatant was pipetted carefully while the rosetted cells were attached to the wall of the tube by the concentrator. The tube was removed from the concentrator, after which 1 ml of washing buffer (PBS/0.1% BSA) was added and the rosetted cells gently resuspended. The tube was repositioned into the concentrator and the supernatant removed. The rosetted cells were washed three times and then resuspended in 250 µl of nuclease-free water and stored at -20°C until needed.

2.2.2 Preparation of saliva samples

Whole mouth saliva (WMS) and parotid saliva (PS) specimens were collected into 50 ml sterile centrifuge tubes (Sarstedt Ltd, Nümbrecht, Germany). WMS was collected, unstimulated, by having the study individuals dribble into the sterile centrifuge tubes. The cellular and supernate fractions of WMS (WMS^c and WMS^s, respectively) were separated by low-speed centrifugation at 1500 rpm for 8 min. The cellular fraction was then resuspended in 250 µl PBS. Both WMS^c and WMS^s were then stored separately at -20°C until needed. PS was collected using small buccal collection cups (Lashley's cup) over the parotid duct and saliva stimulated with 5% citric acid fluid

dropped onto the tongue. The collected parotid saliva was then stored at -20°C until needed.

2.2.3 Preparation of oral brush samples

Cells from the buccal mucosa and palate from the study individuals were collected using cytology sampling brushes (Cytotak® / Medical Wire and Equipment Co., Corsham, Wiltshire, UK). The brush was rotated 10 times against the oral mucosa then placed in a sterile container containing 5 ml PBS. The container was then placed on the vortex. The brush was removed after vortexing. The container and its content were then centrifuged at 3000 rpm for 10 min. Four ml of the supernatant was removed and discarded. The pellet along with the remaining 1 ml of PBS was then stored at -20°C until needed.

2.2.4 Preparation of Biopsy samples

Paraffin-embedded tissue from KS biopsy samples were sectioned using a sledge microtome. Sectioning was carried out by a histopathologist at the Riyadh Armed Forces Hospital. Serial 10-µm sections were folded and inserted in 2 ml Eppendorf tubes (Sarstedt Ltd, Nümbrecht, Germany).

Table 2.2 Cluster of Differentiation (CD) markers on the cell surfaces

CD Antigen	Other names	M. W.	Cell types expressing the antigen	Functions
CD2	T11, LFA-2	45-58	Thymocytes (95%), mature peripheral T cells (almost all), NK cells (80-90%), thymic B cells (50%)	Adhesion molecule, binding CD58 (LFA-3); can activate T cells
CD14		53-55	Macrophages/Monocytes (90%), granulocytes (30%), langerhans cells, dendritic cells, B cells	Receptor for complex of LPS and LPS binding protein
CD19		95	Pre B, B cells, follicular dendritic cells	Co- receptor for B cells
CD31	Pecam-1	130-140	Endothelium, platelets, macrophages, Kupffer cells, granulocytes, T/NK Cells, lymphocytes, megakaryocytes, fibroblasts, osteoclasts, neutrophils	Possibly an adhesion molecule
CD45	Leukocyte Common Antigen (LCA), T200, B220	180-240	All haemopoietic cells, stronger in lymphocytes	Tyrosine phosphatase, augments signalling through antigen receptor of B and T cells

From: 1st International Workshop and Conference on Human Leukocyte Differentiation Antigens (HLDA)

2.3 Amplification of HHV-8 sub-genomic DNA

2.3.1 Laboratory accommodation

PCR–related procedures were performed in four separate laboratory areas in the Virus Reference Division of the Health Protection Agency's Center for Infections, London:

- An extraction room used for processing clinical samples
- A clean room used for preparing reagents (except DNA)
- An amplification room used to run the PCRs
- A gel room for the detection of PCR amplicons by gel electrophoresis

The workflow was strictly unidirectional in order to prevent contamination with PCR products.

2.3.2 DNA Extractions

DNA was extracted from 200 µl of the resuspended dynabeads, brushed buccal, brushed palatal, and saliva cellular and supernatant samples using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Venlo, The Netherlands) following the manufacturer's instruction. The DNA was eluted in 50 µl of Elution Buffer AE (10mM Tris-CL; 0.5 mM EDTA; pH 9.0). Paraffin-embedded tissue from KS biopsy samples were sent to GenoVar diagnostics Ltd (GenoVar Diagnostics Ltd., Sittingbourne, Kent, UK) which uses their in-house nucleic acid extraction method applicable to paraffin-embedded tissue. This method has been specifically designed to extract total nucleic acid from formalin-fixed, paraffin-embedded tissue, and is optimised for extraction of DNA from one section of the tissue of up to 20 µm thickness.

2.3.3 PCR amplification of β–globin DNA

The presence of DNA in the extracts was verified from a number of samples randomly selected from each group. This was done by the amplification and detection of a 268-bp fragment of the housekeeping β-globin gene as an indicator for the

successful extraction. The PCR was carried out in a 25-µl reaction mixture containing nuclease-free water, PCR buffer (200 mM Tris-HCL (pH 8.4), 500mM KCl), 1.5 mM $MgCl_2$, 10 mM each dNTP's, 1 unit *Taq* DNA polymerase (Invitrogen, CA, USA), 20 pmol of each *β*-globin primer (PC04 and GH20) and 2 µl extracted DNA. Single round PCR was then applied. The sequences of the *β*-globin primers were as follows:

PC04: 5`-CAACTTCATCCACGTTCACC-3`
GH20: 5`-GAAGAGCCAAGGACAGGTAC-3`

The thermal cycler (Biometra T3 Thermocycler, Germany) was programmed to 94°C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55°C for 1 min, and 72°C for 1 min, and finally a 5-min extension period at 72°C (Gravitt *et al.*, 1998; Fredricks and Relman, 1999; Sato *et al.*, 2001).

2.3.4 PCR amplification of the HHV-8 ORF 26 DNA

First-round PCR amplification of HHV-8 ORF 26 (hereafter referred to as "KS330") was carried out in a 25-µl reaction mixture containing nuclease-free water, PCR buffer (200 mM Tris-HCL (pH 8.4), 500mM KCl), 4.0 mM $MgCl_2$, 10 mM each dNTP, 1 unit *Taq* DNA polymerase (Invitrogen, CA, USA), 20 pmol of each first round primer (sequence shown below) and 4 µl extracted DNA. The second-round PCR mix was identical to the first except 20 pmol of each inner primer (sequence shown below) was used and 2 µl of the first round product was added as template. The sequences of HHV-8 ORF-26 primers were as follows (Di Alberti *et al.*, 1997b):

KS-1 (26): 5`-AGCCGAAAGGATTCCACCAT-3`
KS-2 (26): 5`-TCCGTGTTGTCTACGTCCAG-3`
KS-inn 1 (26): 5`-TTCCACCATTGTGCTCGAAT-3`
KS-inn 2 (26): 5`-TACGTCCAGACGATATGTGC-3`

The PCR thermocycling conditions for the amplification of the region HHV-8 ORF 26 include heating the samples to 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by a 5-min extension period at

72°C. PCR using outer HHV8 ORF-26 primers produces a product 233 bp in length from positions 355 to 588 and using the inner primers produces a 211-bp product from position 366 to 577. Nucleotide positions are numbered according to the sequence deposited in GenBank under accession number U75698.

2.3.5 PCR amplification of the HHV-8 ORF-K1 DNA

2.3.5.1 K1/V1

First-round PCR amplification of the first variable region (VR1) of HHV-8 ORF K1 (hereafter referred to as "K1/V1") was carried out in a 50-µl reaction mixture containing nuclease-free water, Stratagene buffer number 8 (containing 35 mM $MgCl_2$, 100 mM-Tris HCL (pH 8.8), 750mM KCl) (Stratagene, Amsterdam, The Netherlands), 10 mM each dNTP, 1 unit *Taq* DNA polymerase (Invitrogen, CA, USA) and 20 pmol each outer primer (sequence shown below). To this mixture, 10 µl of extracted DNA was added. The second-round PCR mix was identical to the first except 20 pmol of each inner primer (sequence shown below) was used and 2 µl of the primary product was added as template. The sequences of the HHV-8 K1/V1 primers were as follows (Cook *et al.*, 2002b):

K1 inn 5: 5`-CCCTGGAGTGATTTCAACGC-3`
K1 inn 6: 5`-ACATGCTGACCACAAGTGAC-3`
K1-1: 5`-GAGTGATTTCAACGCCTTAC-3`
K1-N: 5`-TGCTGACCACAAGTGACTGT-3`

The PCR thermocycling conditions for the amplification of the region HHV-8 ORF K1/V1 include heating the samples to 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and finally 5 min at 72°C.

PCR using the ORF K1 outer primers produces a 255-bp fragment from positions 568 to 823, while PCR using the inner primers produces a 247-bp fragment from positions 573 to 820 in the BCBL-1 K1 sequence. Nucleotide positions are numbered

according to the sequence deposited in GenBank (accession number U86667).

2.3.5.2 Long K1

A selected number of HHV-8 K1/V1 or KS330 DNA positive samples were amplified for a longer region of HHV-8 ORF K1 ("long K1") by nested PCR. This long K1 region included both HHV-8 ORF K1 variable regions 1 and 2 (VR1 & VR2). First-round PCR amplification of long K1 was carried out using JumpStart REDTaq ReadyMix PCR Reaction Mix (Sigma, St. Louis, MO, USA). The mix contains JumpStart Taq DNA Polymerase, 99% pure dNTPs, buffer, and an inert dye in a 2x optimized reaction concentrate. 25 µl of this mixture was added to 20 pmol of each outer primer (sequence shown below), 10 µl of extracted DNA, and nuclease-free water to a final reaction volume of 50 µl. The second-round PCR mix was identical to the first except 20 pmol of each inner primer (sequence shown below) was used and 5 µl of the first-round product was added as template. The sequences of the HHV-8 long K1 primers (Cook *et al.*, 1999; Whitby *et al.*, 2004) are as follows:

K1a-f:	5`-ATGTTCCTGTATGTTGTCTGC-3`
K1a-r:	5`-AGTACCAATCCACTGGTTGCG -3`
K1b-f:	5`-GTCTGCAGTCTGGCGGTTTGC -3`
K1b-r:	5`-CTGGTTGCGTATAGTCTTCCG -3`

The PCR thermocycling conditions for the amplification of the HHV-8 long K1 region include heating the samples to 95°C for 1 min 45 s, followed by 35 cycles of 96°C for 1 min, 51°C for 45 s, and 72°C for 1 min. The annealing temperature for the second-round assay was 58°C for 30 cycles. Both assays ended with a 5-min extension period at 72°C (Mbulaiteye *et al.*, 2006). PCR using the long K1 outer primers produces a 867-bp fragment from positions 485 to 1352, while PCR using the inner primers produces a 840-bp fragment from positions 500 to 1340 in the BCBL-1 K1 sequence. Nucleotide positions are numbered according to the sequence deposited in GenBank under accession number U86667.

2.3.6 Detection of the PCR product

Eight µl of the PCR product was mixed with 2 µl of Blue/Orange 6X loading dye (Promega, Wisconsin, USA) and electrophoresed through a 2% agarose gel along with 1 µg of 1-kb ladder molecular weight marker (Invitrogen, CA, USA) on either side of the test samples to assess the size of the amplified product. Positive and negative controls were also added. Electrophoresis took place in a 1X TRIS-borate EDTA buffer (TBE supplied as 10X stock) (Invitrogen, CA, USA). The gels were then stained with ethidium bromide solution (Invitrogen, CA, USA) in TBE buffer (concentration of 5µg/ml). DNA fragments were visualized using a short wave ultra-violet trans-illuminator (Biorad Gel doc System) and photographed using a photo printer (Mitsubishi P91 Video Copy Processor).

2.3.7 Procedures to minimize contamination

To minimize contamination during PCR, DNA extraction, PCR reagent preparation, thermocycling and post-PCR procedures were conducted in dedicated rooms. Appropriate negative control specimens were also included in each PCR. In addition, DNA extraction and PCR were repeated for those samples showing intra-individual HHV-8 subgenomic sequence variation.

2.3.8 Purification of PCR products for sequencing

In order to efficiently recover DNA and remove gel, primers and free nucleotides, which can interfere with subsequent downstream applications, the PCR products were purified directly from the second-round PCR product using GE GFX PCR DNA Purification kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) following the manufacturer's instructions, or from agarose gels by the use of the Qiagen QIAquick Gel Extraction kit (Qiagen Inc., Venlo, The Netherlands). The latter technique involved loading the PCR products onto a 2% composite agarose gel and electrophoresis, as described in Section 2.3.6. Alternate wells were used to allow

adequate area for cutting out each band from the gel without risk of contamination from other products. Short wave transillumination was used to visualize the bands so that they could be carefully cut out with a scalpel, and transferred to separate 1.5 ml Eppendorfs. The Eppendorfs were then weighed and the appropriate amount of buffer QG (containing a pH indicator) added. Buffer QG solubilizes the agarose gel slice and provides the appropriate conditions for binding of DNA to the silica membrane in preparation for DNA purification, as detailed by the manufacturer's instructions.

In addition, plasmid DNA was purified using Qiagen QIAprep Spin Miniprep Kit (Qiagen Inc., Venlo, The Netherlands) when needed. This technique involves alkaline lysis of bacterial cells followed by adsorption of DNA onto a silica membrane in the presence of high salt in an attempt to obtain an optimum combination of DNA quality, yield and concentration.

To confirm that the purification had been successful and to estimate the quantity of DNA recovered, 1 µl of the supernatant was taken and run on a 2% agarose gel alongside a 1µg of 1-kb ladder (Invitrogen, CA, USA), stained and visualised before subsequent cloning or sequencing.

2.3.9 Cloning

Second-round K1/V1 PCR products of some positive samples were cloned prior to DNA sequencing using the TOPO TA cloning kit (Invitrogen). This resulted in better quality sequencing results, and allowed multiple clones from one sample to be compared, thereby ensuring sequence fidelity.

The PCR4–TOPO vector is supplied linearised with the addition of a 3' thymidine (T) overhang to complement the 3' terminal A of the PCR product. During PCR, Taq

polymerase adds a single adenosine (A) to 3' end of the PCR product through its non-template dependent terminal transferase activity (Marchuk *et al.*, 1991; Shuman, 1994). Ligation of vector and PCR product occurs through the activity of topoisomerase I, covalently bound to the vector (Shuman, 1994). Chemically competent *E. coli* are used to transform the cloning reaction and are grown overnight on L agar (Luria agar: 1.0% tryptone, 5.0% yeast extract, 1.0% 0.17 M NaCl, 1.5% agar, pH 7.0) containing 50 mg/ml ampicillin. PCR using second-round primers was performed directly on the resulting colonies and prior to sequencing.

2.3.10 DNA sequencing

The samples were sequenced using the Beckman CEQ2000 automated capillary array sequencer. Purified DNA was added to the PCR sequencing reaction consisting of the following components: 5 µl of sterile water, 3 µl of inner PCR primers (2 pmol), and 8 µl of Beckman Coulter sequencing kit mix (Beckman Coulter Inc, CA, USA), to a total volume of 20 µl. Sequencing PCR reactions were purified using the CleanSEQ® Sequencing Reaction Clean-Up System (Agencourt Bioscience Corp., MA, U.S.A). CleanSEQ® contains magnetic particles in an optimized binding buffer to selectively capture sequencing extension products.

The CleanSEQ® procedure (Figure 2.1) is performed in three stages:

1. Selective binding of sequencing extension products to paramagnetic beads and separation of the beads with a magnetic field;
2. Washing of the beads with 85% ethanol to remove unincorporated dyes, nucleotides, salts and other contaminants;
3. Elution of the purified sequencing product from the paramagnetic beads using formamide.

Figure 2.1 CleanSEQ® Process overview

1. Agencourt CleanSEQ reagent and ethanol addition to sequencing reaction; 2. sequencing products binding to magnetic beads; 3. Separation of sequencing products from contaminants with magnetic field; 4. Washing with ethanol; 5. Elution from magnetic particles 6. Transfer away from magnetic beads.

[Adapted from: http://www.agencourt.com/poducts/spri_reagents/cleanseq]

The sequencing products were then immediately loaded onto the automated sequencer. The Beckman CEQ-2000 uses a capillary system to electrophorese the samples through a polyacrylamide gel contained in a capillary tube. In the sequencing PCR mix, chain terminating nucleotide bases with different fluorescent tags are present and are incorporated into the DNA during PCR amplification. During electrophoresis a laser reads these fluorescent bases to determine the sequence of the sample.

2.3.11 Analysis of sequence data

Raw chromatograph data were analysed using the SeqMan sequence analysis software, and multiple alignments were made in Megalign. Both programmes were from the LASERGENE sequence analysis package (DNAstar Inc., Madison, WI, USA). The Megalign programme allowed the degree of similarity between different sequences to be analysed by comparing nucleotides and/or amino acids. The alignments were viewed in four different formats: the Alignment Report, Sequence Distances, Residue Substitution and Phylogenetic Tree using the UPGMA method of clustering analysis. Genetic distances were expressed as a percent nucleotide divergence over the entire tree. Further analysis was performed after all sequence data had been collected. Alignments were entered into the *PHYL*ogeny *I*nference *P*ackage (PHYLIP version 3.6, Seattle, USA). The following programmes were used to analyse sequence data in PHYLIP: DNADIST and NEIGHBOR. Alignments were first analysed using SEQBOOT to create 1000 multiple data sets, resampled from the input data set using random sampling methods with replacement. Trees generated in SEQBOOT were entered into the CONSENSE program and the branching patterns that occurred most frequently were reflected on the consensus tree. Values at each node indicated the percentage of trees containing each branching pattern and could be used to interpret the robustness of the resulting tree.

2.4 Quantitative PCR

Selected WMS[s] extracts, showing positive results for K1/V1 and KS330 DNA, were sent to Dr S Dollard, the National Centre for Infectious Diseases, Centres for Disease Control and Prevention, Atlanta, GA, where a quantitative, fluorescence-based, real-time PCR assay targeting ORF 25, was applied as previously described (Stamey *et al.*, 2001). Each 50 µl reaction contained extracted DNA, 600 nM each primer, and 200 nM probe. The samples were tested in duplicate.

2.5 Serological detection of HHV-8

An ELISA (Advanced Biotechnologies Inc., Columbia, MD, USA) was used for the detection of HHV-8 IgG antibodies in the participants' plasma samples. This uses, as antigen, a whole viral extract from the KS-1 cell line which was derived from a PEL of an EBV/HIV-uninfected patient. The test detects antibody to the majority of HHV-8 structural proteins (Said *et al.*, 1996b; Chatlynne *et al.*, 1998).

The plasma was first diluted in a provided sample diluent (1:101), and 100 µl applied to each well, with the positive control (in duplicate), the negative control (in triplicate) and the reagent blank (100-µl sample diluent) in another well. The test procedure involves three incubation steps: In the first, the plasma (at a 1:101 dilution) is incubated in the antigen-coated microtitre wells. The wells are then washed to remove any unbound sample components. The second step involves the addition of horseradish peroxidase conjugated anti-human IgG (Fc-specific) to the wells. The conjugate binds to the antibody immobilized on the wells. The wells are then washed to remove unreacted conjugate. The final incubation step involves the incubation of the microtitre wells containing immobilized peroxidase conjugate with a peroxidase substrate (tetramethylbenzidine). Enzyme-mediated cleavage of the substrate results in a colour change of positive samples to blue. After 30 min the reaction is stopped by adding a sulphuric acid stop solution. The colour of the positive samples then

changes from blue to yellow, and the colour intensity is measured spectrophotometrically. The colour intensity of the solution is proportional to the antibody concentration in the test sample. Optical Density (OD) ratios were calculated by dividing the reading of each sample well by the cut-off value (the average of the three negative control readings multiplied by 3). The OD ratios were then interpreted as follows:

	OD Ratio
Negative samples	≤0.75
Positive samples	≥1.00
Equivocal (borderline)	0.76 – 0.99

2.6 Screening for inter- and intra- sample K1/V1 sequence differences by denaturing gradient gel electrophoresis (DGGE)

2.6.1 K1/V1 Expand high-fidelity PCR and cloning

Selected samples that amplified positively for K1/V1 were subjected again to nested PCR using same conditions as in Section 2.3.5.1 except that 0.7 units of the EXPAND High Fidelity PCR System (Roche Diagnostics, Penzberg, Germany) were used instead of *Taq* DNA polymerase. The PCR products were then purified directly from the second-round PCR product using GFX PCR DNA Purification kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) following the manufacturer's instructions. Clones were generated from each K1/V1 PCR product by use of the TOPO TA Cloning System (Invitrogen, CA, USA) as detailed in Section 2.3.9.

2.6.2 GC-clamp colony PCR

For at least 15 clones from each amplificate, another round of PCR was done under conditions identical to the second-round high-fidelity PCR, except that a "clamping" primer was used in place of the forward inner sense primer. The clamping primer contains a guanine-cytosine rich domain with a high melting temperature, to prevent

complete denaturation of the PCR product (Sheffield *et al.*, 1989; Woodward *et al.*, 1994). This primer, K1-1 clamp, has the following sequence:

5'CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGAGTGATTTCA ACGC-3'

The sequence for the reverse inner sense primer is as described in Section 2.3.5.1. Colony PCR products were separated and visualised on an agarose gel as described in Section 2.3.6.

2.6.3 Preparing denaturing gradient polyacrylamide gels

Stock solutions of each acrylamide mix were prepared beforehand and cooled to 4°C prior to use. For the K1/V1 colony PCR product, a gel gradient of 30% to 50% denaturants was necessary to achieve discrimination to a 1 base pair mutation. The 50% solution contained 10% acrylamide, 0.6% TAE buffer, 20% formamide and 3.5 M urea. The 30% solution contained 10% acrylamide, 0.6X TAE, 12% formamide, and 2.1 M urea. Using a gravity-driven gradient maker with pump drive (GRI, Braintree, UK), the gradient gel was poured using 30 ml of each solution. To allow the gel to polymerise 13 µl of N, N, N', N' -tetramethylethylenediamine (TEMED) (Invitrogen) and 250 µl of 10% ammonium persulfate per each 30 ml of stock solution were added prior to pouring. A gradient gel was pumped between glass plates. A 48-well comb was inserted and the gel was left to polymerise for at least 6 h.

2.6.4 Loading and electrophoresis

Clamped colony PCR products (4 µl) generated as described in Section 2.6.2 were mixed with 2 µl of Blue/Orange 6X loading dye (Promega, Wisconsin, USA). After all the samples were loaded into the wells, electrophoresis in 0.6X TAE buffer at 60°C and 100 V took place over 16 h. The electrophoresis apparatus allowed for continuous flow of buffer to maintain a constant temperature of 60°C throughout the

run (Igeny PhorU).

2.6.5 Staining of gels

Gels were stained in a solution of ethidium bromide (Invitrogen) in 200 ml of 0.6X TAE buffer (concentration of 1.25µg/ml) for 1 h. Bands were visualised by a short wave ultraviolet transilluminator (Biorad Gel doc System) and photographed using a photo printer (Mitsubishi P91 Video Copy Processor). K1/V1 inserts in clones corresponding to bands that migrate to disparate gel positions were sequenced for genotypic and subgenotypic differences as described in Section 2.3.10 and raw chromatograph data analysed as described in Section 2.3.11.

2.7 Screening for inter- and intra- sample long K1 sequence differences by cloning and DNA sequencing

2.7.1 Isolation of DNA band from agarose gel

Selected PCR products that amplified positively for long K1 were loaded onto a 2% composite agarose gel and underwent electrophoresis. The bands were carefully cut out with a scalpel, and transferred to separate 1.5 ml Eppendorfs, after which DNA purification was performed using Qiagen QIAquick Gel Extraction kit (Qiagen) as described in Section 2.3.8. Purified DNA fragments were visually verified by gel examination before cloning.

2.7.2 Cloning

The long K1 gene fragment was cloned into vectors by use of the TOPO-TA Cloning® kit (Invitrogen, CA, USA) as described in Section 2.3.9. Colonies were screened by PCR using HHV-8 long K1 second-round primers as described in Section 2.3.5.2. DNA of at least ten clones of each amplificate was purified using GE GFX PCR DNA purification kit or Qiagen QIAprep Spin Miniprep Kit when needed. Purified DNA fragments were visually verified by gel examination before sequencing.

2.7.3 Sequencing

The selected clones were sequenced using forward and reverse M13 and long K1 inner primers. Sequencing was performed using the Beckman-Coulter CEQ2000 automated capillary array sequencer as described in Section 2.3.10. Raw DNA sequence data were analyzed using Seqman software (DNAstar) (Section 2.3.11).

2.8 Statistical analysis

Data entry and analysis were performed using the SPSS for Windows (Statistical Package of Social Science) software, version 12.0. Frequency distributions and cross-tabulation tables were constructed in order to analyze the demographic, clinical and virological information. In an attempt to detect possible risk factors, both logistic regression and chi-squared testing (the *P* value for statistical significance was set at <0.05) were used. The independent samples *t*-test, ANOVA and Bivariate correlation (Spearman's rank correlation) were used as appropriate.

Chapter 3

HHV-8 Geno- and Sero-prevalence in the General Population, Patients with Renal Failure, and Renal Allograft Recipients

3.1 Introduction

Serological studies reveal that the prevalence of HHV-8 in populations varies widely throughout the world (Whitby *et al.*, 1995; Gao *et al.*, 1996b; Kasolo *et al.*, 1997; Whitby *et al.*, 1998; Rezza *et al.*, 1998; Olsen *et al.*, 1998; Perna *et al.*, 2000; Iscovich *et al.*, 2000; Chokunonga *et al.*, 2000; Schulz, 2000a; He *et al.*, 2007). The prevalence of KS also varies widely. With regard to post-transplantation KS, the rate of KS among solid-organ transplant recipients varies by geographic location. Generally, countries with higher rates of seropositivity to HHV-8 also have higher rates of KS among the transplant population. However, this situation does not seem to apply to Saudi Arabia, where KS is particularly prevalent among renal allograft recipients compared to other geographic regions. Previous studies have suggested that HHV-8 seroprevalence is relatively low (ranging between 1.7% and 7%) in the general Saudi Arabian population (Qunibi *et al.*, 1998; Almuneef *et al.*, 2001; Alzahrani *et al.*, 2005), yet high (ranging between 18% and 28%) in renal allograft recipients without KS (Qunibi *et al.*, 1998; Alzahrani *et al.*, 2005). Such a disparity in HHV-8 prevalence rates between the general population and renal transplant recipients has not been reported elsewhere and the reason for the particularly high incidence of post-transplantation KS in this geographic area is unknown.

Saudi Arabian patients with ESRD, unlike those from other regions (Cattani *et al.*, 2001) were reported to have an increased risk of HHV-8 infection (7% to 29%) (Qunibi *et al.*, 1998; Almuneef *et al.*, 2001) compared with subjects without renal disease (ranging between 1.7% and 7%) (Qunibi *et al.*, 1998; Almuneef *et al.*, 2001; Alzahrani *et al.*, 2005). The finding by Almuneef *et al.* (2001), in the later and larger study, of antibodies to HHV-8 in only 7% of ESRD patients is comparable in magnitude to the incidence reported in patients in Greece receiving haemodialysis (7.2%) (Zavitsanou *et al.*, 2006).

Most previous studies of orally shed HHV-8 have been conducted in Western gay men and in people who live in KS-hyperendemic areas such as Africa. No studies appear to have involved patients with renal disease in another group at particular risk of KS – those from the Middle East (Penn, 1983; El Agroudy *et al.*, 2003).

Given the issues outlined above with regard to HHV-8 infection and disease relating to post solid-organ transplant patients in Saudi Arabia, an investigation into the extent of serologic as well as genomic prevalences of HHV-8 infection in the Saudi Arabian general population, patients with end-stage renal disease receiving haemodialysis, and renal allograft recipients was conducted. Additionally, the shedding of HHV-8 via saliva and the possibility of HHV-8 infection in different anatomical compartments in patients with renal disease was determined to define the most likely sites of HHV-8 shedding.

3.2 Patients and samples

3.2.1 Study population

Samples were collected from four different groups of participants (BD, PW, CRF and RAR) attending the Armed Forces Hospital in Riyadh, Saudi Arabia (Table 3.1). All participants were of Saudi nationality. Clinical data on BD, PW, CRF and RAR patients were obtained from computer-based and standard medical records and documented onto standardized forms, which were linked to patient samples by numerical code. The clinical, virological and immunological characteristics determined for each of the four groups are listed in Table 2.1. Oral findings and indices were documented using a standard proforma (Appendix 1).

Table 3.1 Demographic features of the four study groups

Group	n	Male	Female	Age			
				mean (yr)	SD	median (yr)	range (yr)
BD	178	176	2	30	7	30	18 - 51
PW	60	0	60	27	7	28	17 - 43
CRF	72	43	29	57	14	60	23 - 83
RAR	61	37	24	40	13	41	19 - 72

BD, blood donors; PW, pregnant women; CRF, patients with chronic renal failure; RAR, renal allograft recipients with no history of KS

3.2.2 Sample collection

Blood samples were obtained from the BD and PW groups as described in Section 2.2.1, while matched oral and blood samples were obtained from the CRF and RAR groups as described in Sections 2.2.1, 2.2.2 and 2.2.3.

3.2.3 Sample processing

After separation of plasma from the blood, the CD45+, CD31+ and CD19+ cell subsets were immunomagnetically fractionated as described in Sections 2.2.1.1 and 2.2.1.2. The cellular and supernate fractions of WMS (WMS^c and WMS^s, respectively) were separated by low-speed centrifugation. BE and PE were similarly pelleted. Following aspiration of the supernate, WMF^c, BE and PE were resuspended in 1 ml of PBS, and stored at -20°C until required. DNA was extracted from blood cell subsets and oral samples as described in Section 2.3.2. The presence of DNA in randomly selected extracts was verified by amplifying a fragment of the β-globin gene as described in Section 2.3.3.

3.2.4 Anti HHV-8 IgG detection

The Advanced Biotechnologies ELISA was applied to all plasma samples as detailed in Section 2.5.

3.2.5 Sequencing analysis of DNA amplified from KS330 and K1

The 211-bp KS330 segment was amplified from sample extracts by use of nested PCR (Section 2.3.4). The 247-bp K1/V1 segment was amplified from sample extracts by nested PCR (Section 2.3.5.1). PCR products were sequenced, raw DNA sequence data analyzed and phylogenetic analyses performed as detailed in Sections 2.3.10 & 2.3.11.

3.2.6 Study of demographic, clinical and virological characteristics

Data entry and analysis were performed using the SPSS for Windows (Statistical Package of Social Science) software, version 12.0. Frequency distributions and cross-tabulation tables were constructed in order to analyze the demographic, clinical and virological information. In an attempt to detect possible risk factors, both logistic regression and chi-squared testing (the *P* value for statistical significance was set at <0.05) were used to determine the effect of general and specific characteristics on HHV-8 genoprevalence, seroprevalence and K1/V1 genotypes. When investigating potential differences in age between groups, the independent samples *t*-test and ANOVA were used as appropriate. Bivariate correlation (Spearman's rank correlation) was used to test for WMS^S viral load interaction with oral health.

The general and specific variables examined are listed below:

3.2.6.1 General Variables

Age, gender, area of origin (area of birth and childhood according to the administrative area; Fig 3.1), viral infections (hepatitis B and hepatitis C) and oral indices (plaque index, gingival index and bleeding index).

3.2.6.2 Specific Variables

For the CRF group, these were history of previous transplant and duration of dialysis. For the RAR group, these were history of previous transplant, type of renal allograft, nationality of allograft donor, relation of donor, location of transplant, time elapsed since transplant, and medications administered (ciclosporin, prednisolone, tacrolimus, mycophenolate mofetil and antivirals).

Figure 3.1 Map of Saudi Arabia showing the 13 administrative regions

[Adapted from: http://www.geocities.com/brianjamesdunn/saudi4.jpg]

3.2.7 Quantitative PCR

All WMS[s], showing positive results for both K1/V1 and KS330 DNA, were referred to the National Centre for Infectious Diseases, Centres for Disease Control and Prevention, Atlanta, GA, where a quantitative, fluorescence-based, real-time PCR was applied as described in Section 2.4.

3.2.8 Investigation of Taq polymerase-induced misincorporation

In this study, 1239 samples were tested by nested PCR for 2 HHV-8 ORFs. *Taq* polymerase was the preferred polymerase used for PCR, being the most cost-effective for large-scale reactions. However, *Taq* polymerase does not have 3' to 5' exonuclease activity and cannot therefore excise misincorporated nucleotides during polymerisation. This may lead to the presence of artefactual mutation errors in a proportion of the PCR amplicons. It has been estimated that *Taq* polymerase will introduce an error at a rate of approximately 0.85×10^{-4} errors/base pair/cycle.

In order to estimate the impact of *Taq* polymerase-induced mutations on the clonal diversity displayed by DGGE, clones produced from an ORF K1/V1 PCR product, amplified from the HHV-8 infected cell line, BCBL-1, were analysed. From each PCR product amplified using either *Taq* polymerase or the EXPAND system, 11 clones were produced and amplified in colony PCR. Previous single-strand conformational polymorphism (SSCP) studies on BCBL-1 K1/V1 clones, amplified using the high-fidelity EXPAND DNA polymerase system, had indicated no viral diversity was present in this cell line (C. Bez, personal communication). EXPAND contains a mixture of both *Taq* polymerase and *Pwo* polymerase. *Pwo* polymerase possesses 3' to 5' exonuclease proofreading activity and as a result the EXPAND system will produce errors at a rate of only 8.5×10^{-6} errors/base pair/cycle. Another 11 clones using Taq polymerase to amplify ORF K1/V1 PCR product followed by the use of EXPAND during the screening PCR (colony PCR) were also examined.

3.2.9 Intra-person K1/V1 sequence differences as revealed by DGGE

Two CRF patients (CRF24 and CRF57), from whom non-identical ORF K1/V1 sequences were recovered and K1/V1 amplification using EXPAND was possible, were chosen for DGGE analysis. From CRF 24 and CRF 57, oral and blood samples, which amplified positively for both examined regions, were subjected again to nested PCR by use of the EXPAND (Section 2.6.1). The PCR products were then purified directly from the second-round product (Section 2.3.8) and clones generated (Section 2.3.9). From approximately 22 colonies from each amplificate, another round of PCR was done using a clamping primer as described in Section 2.6.2, and the products were visualised by agarose gel electrophoresis. The PCR products from 16 selected colonies were subjected to DGGE as detailed in Sections 2.6.3, 2.6.4 and 2.6.5, followed by nucleotide sequencing (Section 2.3.10).

3.3 Results

3.3.1 Anti HHV-8 detection

IgG antibodies to HHV-8 were detected in the plasma of one person in the BD group (0.6%) (Table 3.2), none in the PW group, 12 in the CRF group (16.7%) (Table 3.3), and four in the RAR group (6.6%) (P <0.001, Pearson Chi-square) (Table 3.5). Although the Chi-square test results showed that there was a statistical significant difference, this result should be treated with caution, as the number of cells employed in the statistical analysis, with an expected count less than 5 was 37.5%.

HHV-8 IgG was found significantly more frequently in CRF patients (12/72; 16.7%) than in healthy control individuals (1/238; 0.4%) (P <0.001, Fisher's exact test). HHV-8 IgG was also found to be more frequent in renal transplant recipients (4/61; 6.6%) than in healthy control individuals (1/238; 0.4%) (P = 0.007, Fisher's exact test). However, no significant difference in HHV-8 IgG was found between CRF and RAR patients (P = 0.108, Fisher's exact test)

3.3.2 HHV-8 DNA detection

The rate of HHV8-subgenomic DNA detectability varied according to the sample group, type of sample collected (blood, saliva or oral brushes) and the target of amplication within the HHV-8 genome.

3.3.2.1 Blood donors and pregnant women

HHV-8 subgenomic DNA was detected in the CD45+ blood cells from nine blood donors (5.1%) and from none of the pregnant women. All HHV-8-genopositive blood donors were male, with a mean age of 28.7 yr (SD= 7.7) and median age of 29 yr (range: 19 – 40 yr). One donor's blood sample was concordantly positive for KS330 and K1/V1, three were positive for KS330 alone and five were positive for K1/V1 only (Table 3.2).

Table 3.2 Saudi blood donors with HHV-8 geno- or sero-positivity

Donor	Age (yr)	KS330	K1/V1	Anti-HHV-8
1	27	pos	pos	neg
2	40	pos	neg	neg
3	22	neg	pos	neg
4	29	pos	neg	neg
5	29	neg	pos	neg
6	33	neg	pos	neg
7	32	neg	neg	pos
8	39	neg	pos	neg
9	20	neg	pos	neg
10	19	pos	neg	neg

3.3.2.2 Chronic renal failure group

The detectability rates according to the amplifiable subgenomic DNA segments were as follows: concordantly positive for KS330 and K1/V1: 1/72 (1.4%) for CD19+ cells, none for CD31+ cells, 3/72 (4.2%) for CD45+ cells, 5/69 (7.3%) for WMS^s, 5/69 (7.4%) for WMS^c, none for PS, 1/69 (1.5%) for BE, 1/69 (1.5%) for PE; positive for KS330 alone: 1/72 (1.4%) for CD19+ cells, 1/72 (1.4%) for CD31+ cells, none for CD45+ cells, 1/69 (1.5%) for WMS^s, 4/69 (5.8%) for WMS^c, none for PS and for BE, 2/69 (2.9%) for PE; and, positive for K1/V1 alone: 1/72 (1.4%) for CD19+ cells, 8/72 (11.1%) for CD31+ cells, 1/72 (1.4%) for CD45+ cells, 3/69 (4.4 %) for WMS^s, 3/69 (4.4 %) for WMS^c, 2/41 (4.9%) for PS, 5/69 (7.3%) for BE, and 5/69 (7.3%) for PE (Table 3.3). For 4 people, HHV-8 DNA could be amplified from ≥4 samples. For 2 people, HHV-8 DNA could be amplified from three samples. For 4 people, HHV-8 could be amplified from two samples, and for nineteen patients only one sample amplified positively for HHV-8.

For CRF patients from whom both oral and blood samples were collected (n= 69), solitary or concordantly positive results for HHV-8 K1 and/or KS330 DNA were detected in one or more oral samples from 22 (31.9%) patients and in at least one blood sample from 11 (15.9%) patients. HHV-8 DNA was significantly more detectable in the oral samples of CRF patients than in their blood (P = 0.043, McNemar's test). Table 3.4 and Figure 3.2 compare oral (WMS^s, WMS^c, PS, BE and PE) and blood (CD19+, CD31+ and CD45+ cells) HHV-8 detection in CRF patients.

Nearly one third of CRF patients (20/69) were HHV-8 genopositive but not seropositive, while three CRF patients were seropositive but not genopositive (3/69, 4.4%) and nine were both HHV-8 geno- and sero-positive (9/69, 13%) four of whom were positive for HHV-8 DNA in their oral samples only and two positive in their blood samples alone. Three (25%) out of the 12 HHV-8 seropositive CRF patients had

detectable HHV-8 DNA in both their blood and oral compartments. HHV-8 K1/V1 DNA in her blood sample.

Among all CRF HHV-8-antibody positive patients (n=12), 7/12 (58.3%) were solitary or concordantly positive for HHV-8 K1 or/and KS330 DNA PCR in their oral samples and 5/12 (41.7%) were solitary or concordantly positive for HHV-8 K1 or/and KS330 DNA PCR in their blood samples. The results based on the logistic regression analysis (the odds ratios (OR) and their corresponding 95% confidence intervals) revealed that CRF patients with detectable antibodies have 6.1 times the odds of having HHV-8 in blood (95% CI: 2.3, 23.6), and 4 times the odds of having HHV-8 in their oral samples (95% CI: 1.1, 14.2) compared to CRF patients without detectable antibodies.

Table 3.3 ELISA and HHV-8 DNA PCR findings in oral and blood samples of CRF patients

CRF ID number	HHV-8 PCR RESULTS																HHV-8 Seropositivity
	CD19		CD31		CD45		WMS[s]		WMS[c]		PS		BE		PE		
	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	
24	pos	pos	neg	pos	pos	pos	pos	pos	pos	pos	NA	NA	pos	pos	pos	pos	pos
57	pos	neg	neg	neg	pos	pos	pos	pos	pos	pos	neg	neg	neg	neg	neg	pos	pos
56	neg	neg	neg	neg	neg	neg	pos	pos	pos	pos	neg	neg	neg	pos	pos	neg	pos
23	neg	neg	neg	pos	pos	pos	pos	pos	pos	pos	NA	NA	neg	neg	neg	neg	pos
10	neg	neg	neg	neg	neg	neg	pos	pos	pos	pos	NA	NA	neg	neg	neg	pos	pos
43	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	pos	neg	pos	neg	neg	neg
15	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	neg	pos	neg	neg	pos
46	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	NA	NA	neg	neg	neg	neg	pos
11	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	pos
48	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos
44	neg	neg	neg	pos	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg
54	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	pos	neg	neg	neg
37	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg
8	neg	pos	neg	neg	neg	pos	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	neg
2	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	neg
7	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
34	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
65	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
58	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
60	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
61	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg
32	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg
41	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	NA	NA	neg	neg	neg	neg	neg
45	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg
12	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
62	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	pos	neg
9	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	pos	neg
72	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg
69	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg
3	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	pos
33	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	pos
64	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos

CD45+, CD31+ and CD19+: PBMC subsets; WMS[s], whole mouth saliva supernate fraction; WMS[c], whole mouth saliva cellular fraction; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; pos, HHV-8 DNA amplified; neg, HHV-8 DNA not amplified; NA, sample not available

Table 3.4 Oral and blood HHV-8 shedding in CRF patients

	Non-infected No. (%)	Oral infection only No. (%)	Blood infection only No. (%)	Oral and blood infection No. (%)	Total No. (%)
CRF Patients	40 (58)	18 (26)	7 (10)	4 (6)	69 (100)

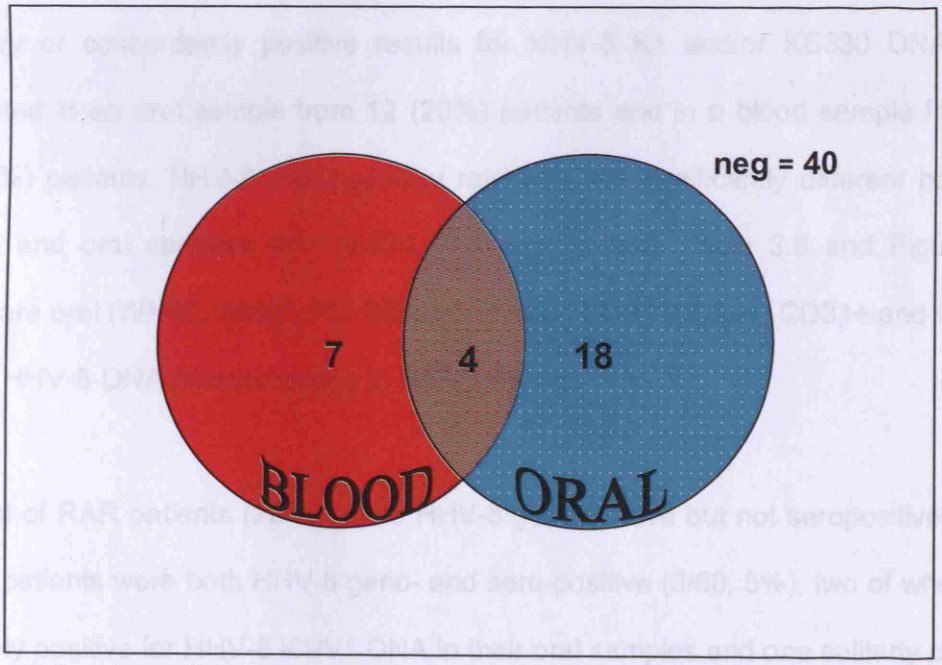


Figure 3.2 Venn diagram illustrating oral and blood shedding of HHV-8 in CRF patients

3.3.2.3 Renal allograft recipient group

The detectability rates according to the amplifiable subgenomic DNA segments were as follows: concordantly positive for KS330 and K1/V1: 2/61 (3.3%) for CD19+ cells, none for CD31+ cells, CD45+ cells, WMS^s, WMS^c, PS, BE and PE; positive for KS330 alone: 1/61 (1.6%) for CD19+ cells, none for CD31+ cells, CD45+ cells, WMS^s, WMS^c, PS, BE and PE; and, positive for K1/V1 alone: 8/61 (13.1%) for CD19+ cells, 1/61 (1.6%) for CD31+ cells, 3/61 (4.9%) for CD45+ cells, 6/60 (10%) for WMS^s, 5/60 (8.3%) for WMS^c, none for PS, 3/60 (5%) for BE, and none for PE (Table 3.5). For one person, HHV-8 DNA could be amplified from three samples. For three people, HHV-8 DNA could be amplified from two samples and for twenty patients only one sample amplified positive for HHV-8.

For RAR patients, from whom both oral and blood samples were collected (n=60), solitary or concordantly positive results for HHV-8 K1 and/or KS330 DNA were detected in an oral sample from 12 (20%) patients and in a blood sample from 14 (23.3%) patients. HHV-8-DNA-positivity rate was not significantly different between blood and oral samples (P = 0.824, McNemar's test). Table 3.6 and Figure 3.3 compare oral (WMS^s, WMS^c, PS, BE and PE) and blood (CD19+, CD31+ and CD45+ cells) HHV-8-DNA detection rates in RAR patients.

A third of RAR patients (20/60) were HHV-8 genopositive but not seropositive, while three patients were both HHV-8 geno- and sero-positive (3/60, 5%), two of who were solitarly positive for HHV-8 K1/V1 DNA in their oral samples and one solitarly positive for HHV-8 K1/V1 DNA in her blood sample.

In this study, 9/178 (5.1%) of CD45+ cell samples from blood donors, none (0/60) from the PW group, 4/72 (5.6%) from the CRF group, 3/61 (4.9%) from the RAR group yielded HHV-8 subgenomic amplicons (P = 0.352, Pearson Chi-square).

Moreover, no statistically significant difference was observed in HHV-8 subgenomic detection rates in CD45+ cell samples between patients with renal disease and healthy control individuals (P = 0.596, Fisher's exact test) or between CRF and RAR patients (P = 1, Fisher's exact test). The detectability rates in the four study groups are summarised in Table 3.7.

Table 3.5 ELISA and HHV-8 DNA PCR findings in oral and blood samples of RAR patients

RAR ID number	HHV-8 PCR RESULTS																HHV-8 Seropositivity
	CD19		CD31		CD45		WMS^s		WMS^c		PS		BE		PE		
	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	
61	neg	neg	neg	neg	neg	neg	neg	pos	neg	pos	NA	NA	neg	pos	neg	neg	pos
10	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	NA	NA	neg	pos	neg	neg	neg
72	neg	neg	neg	neg	neg	pos	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg
17	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	neg
23	neg	pos	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
63	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	pos
58	neg	pos	neg	neg	neg	neg	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	pos
13	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
24	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
66	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	neg
70	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	neg
8	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	neg
9	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
11	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
20	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	NA	NA	neg	neg	neg	neg	neg
31	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
56	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	NA	NA	neg	neg	neg	neg	neg
34	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg
16	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg
36	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	NA	NA	neg	neg	neg	neg	neg
32	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg
27	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	NA	NA	neg	neg	neg	neg	neg
14	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
21	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	pos

CD45+, CD31+ and CD19+: PBMC subsets

WMS^s, whole mouth saliva supernate fraction; WMS^c, whole mouth saliva cellular fraction; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; pos, HHV-8 DNA amplified; neg, HHV-8 DNA not amplified; NA, sample not available

Table 3.6 Oral and blood HHV-8 shedding in RAR patients

	Non-infected No. (%)	Oral infection only No. (%)	Blood infection only No. (%)	Oral and blood infection No. (%)	Total No. (%)
RAR Patients	37 (62)	9 (15)	11(18)	3 (5)	60 (100)

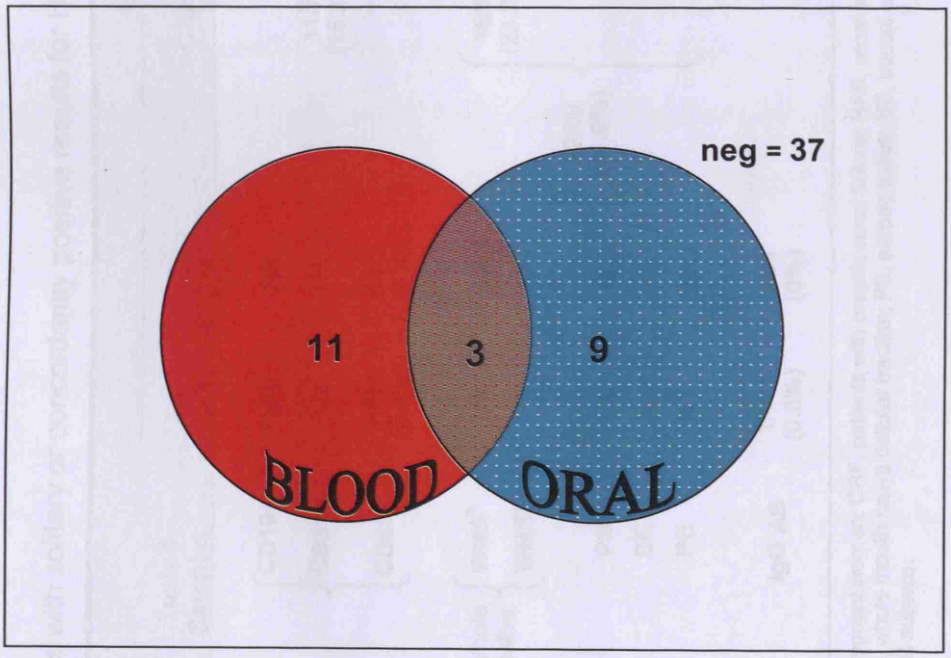


Figure 3.3 Venn diagram illustrating oral and blood shedding of HHV-8 in RAR patients

Table 3.7 Patients with solitary or concordantly positive results for HHV-8 K1/V1 or/and KS330 DNA

	Tested Samples		Group: BD	Group: PW	Group: CRF patients		Group: RAR patients	
Total bbod genoprevalence		CD19+	NA	NA	11/72 (15.3%)	3/72 (4.2%)	15/61 (24.6%)	11/61 (18%)
		CD31+	NA	NA		9/72 (12.5%)		1/61 (1.6%)
		CD45+	9/178 (5.1%)	0/60 (0%)		4/72 (5.6%)		3/61 (4.9%)
Total oral genoprevalence	Whole saliva	WMS[s]	NA	NA	22/69 (31.9%)	16/69 (23.2%) 9/69 (13%)	12/60 (20%)	10/60 (16.7%) 6/60 (10%)
		WMS[c]	NA	NA		12/69 (17.4%)		5/60 (8.3%)
		PS	NA	NA		2/41 (4.9%)		0/33 (0%)
		BE	NA	NA		6/69 (8.7%)		3/60 (5%)
		PE	NA	NA		8/69 (11.6%)		0/60 (0%)
Seroprevalence		IgG AB	1/178 (0.6%)	0/60 (0%)		12/72 (16.7%)		4/61 (6.6%)

BD, blood donors; PW, pregnant women; CRF, patients with chronic renal failure; RAR, renal allograft recipients with no history of KS; WMS[s], whole mouth saliva supernate fraction; WMS[c], whole mouth saliva cellular fraction; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; NA, sample not available; CD45+, CD31+ and CD19+, PBMC subsets.

3.3.3 Demographic, clinical and virological characteristics in relation to HHV-8 geno- and sero-positivity

Demographic characteristics as well as general and specific risk factors in relation to HHV-8 geno- or sero-prevalence are summarised in Tables 3.8, 3.9, 3.10 & 3.11.

3.3.3.1 Gender

All HHV-8 geno- or sero-positive individuals from the combined BD and PW group were male, with 1/176 (0.6%) of the male participants found to be seropositive compared to none of females (P = 1.0, Fisher's exact test) and 9/176 (5%) of males genopositive compared to none of females (P = 0.117, Fisher's exact test).

There was no statistically significant difference in the proportion of seropositive females compared to seropositive males in the CRF group (7/29 [24.1%] vs. 5/43 [11.6%]; P = 0.204, Fisher's exact test), and seropositive females compared to seropositive males in the RAR group (2/24 [8.3%] vs. 2/37 [5.4%]; P = 0.643, Fisher's exact test).

More than half (15/27; 55.6%) of females in the CRF group were found to be HHV-8 genopositive in one or more sample compared to 14/42 (33.3%) of males (P = 0.084, Fisher's exact test). There was no significant difference in HHV-8 genopositivity rate between males (16/36; 44.4%) and females (7/24; 29.2%) in the RAR group (P = 0.285, Fisher's exact test).

3.3.3.2 Age

The single seropositive individual from the combined BD and PW group was 32 yr. All HHV-8 genopositive individuals from the combined BD and PW group were below the age of 40 yr. However, no statistically significant association was observed between the ages in these two groups in terms of geno- or seropositivity.

None of the CRF patients ≤50 yr or ≥81 yr were HHV-8 seropositive. None of the RAR patients ≤40 yr or ≥71 yr were seropositive. Seropositivity rate for all other age groups varied between 22.2% and 33.3% amongst CRF patients and between 5% and 33.3% amongst RAR patients.

Two CRF patients aged between 21 yr and 30 yr were HHV-8 genopositive in at least one sample. Less than 50% of CRF patients aged between 30 yr and 60 yr and more than 61% of CRF patients aged between 61 yr and 80 yr were HHV-8 genopositive. In the RAR group, 8/13 (61.5%) aged between 21 yr and 30 yr were genopositive, while patients aged within the age groups 31-40 yr, 41-50 yr, 51-60 yr and 61-70 yr showed a genopositivity rate between 25% to 40%. No CRF patients older than 80 yr or RAR patients older than 71 yr or younger than 21 yr were genopositive.

3.3.3.3 Area of origin

Anti-HHV-8 IgG was detected in: 4/34 (11.8%) of CRF and 0/17 of RAR patients from Riyadh; 1/7 (14.3%) of CRF and 0/3 of RAR patients from Makkah; 0/2 of RAR patients from Madinah; 1/6 (16.7%) of CRF and 0/10 of RAR patients from Qaseem; 1/1 (100%) of CRF and 0/3 of RAR patients from the Eastern province; 3/9 (33.3%) of CRF and 1/7 (14.3%) of RAR patients from Aseer; 0/1 of CRF patients from Tabouk; 1/5 (20%) of CRF and 0/1 of RAR patients from Hail; 1/6 (16.7%) of CRF and 0/6 of RAR patients from Jizan; 1/3 (33.3%) of RAR patients from Najran; 0/1 of CRF and 1/4 (25%) of RAR patients from Albaha; 0/1 of CRF and 1/2 (50%) of RAR patients from Aljouf; and, 0/1 of CRF and 0/3 of RAR patients born outside the Kingdom of Saudi Arabia.

HHV-8 DNA was detected in at least one sample from: 14/34 (41.2%) of CRF and 8/17 (47.1%) of RAR patients from Riyadh; 2/7 (28.6%) of CRF and 2/3 (66.7%) of RAR patients from Makkah; 1/2 (50%) of RAR patients from Madinah; 1/5 (20%) of

CRF and 4/10 (40%) of RAR patients from Qaseem; 1/1 (100%) of CRF and 1/3 (33.3%) of RAR patients from the Eastern province; 6/7 (85.7%) of CRF and 0/6 of RAR patients from Aseer; 0/1 of CRF patients from Tabouk; 4/5 (80%) of CRF and 0/1 of RAR patients from Hail; 1/6 (16.7%) of CRF and 2/6 (33.3%) of RAR patients from Jizan; 1/3 (33.3%) of RAR patients from Najran; 0/1 of CRF and 3/4 (75%) of RAR patients from Albaha; 0/1 of CRF and 1/2 (50%) of RAR patients from Aljouf; and, 0/1 of CRF and 0/3 of RAR patients born outside the Kingdom of Saudi Arabia.

3.3.3.4 Duration of dialysis

CRF patients receiving haemodialysis for a period of 5 yr or less showed a HHV-8 seropositivity rate of 4/36 (11.1%) compared to 8/36 (22.2%) seropositivity rate amongst patients on haemodialysis for more than 5 yr (P = 0.343, Fisher's exact test). CRF patients receiving haemodialysis for a period of 5 yr or less showed a HHV-8 genopositivity rate of 14/35 (40%) compared to a 15/34 (44.1%) seropositivity rate amongst patients on haemodialysis for more than 5 yr (P = 0.809, Fisher's exact test).

3.3.3.5 Previous renal transplant

Anti-HHV-8 IgG was not detected in any of the CRF (n=8) or RAR patients (n=5) with a history of a previous transplant, compared to 12/64 (18.8%) of CRF (P = 0.337, Fisher's exact test) and 4/56 (7.1%) of RAR patients (P = 1.000, Fisher's exact test), respectively, without a history of a previous transplant.

HHV-8 DNA was detected in 3/8 (37.5%) of CRF and 1/5 (20%) of RAR patients with a history of a previous transplant compared to 26/61 (42.6%) of CRF (P = 1.000, Fisher's exact test) and 22/55 (40%) of RAR patients (P = 0.640, Fisher's exact test), respectively, without a history of a previous transplant.

3.3.3.6 Location of transplant

RAR patients who underwent allograft transplant in the city of Riyadh (KSA) showed a seropositivity rate of 2/17 (11.8%) and the single RAR patient who received the allograft in Jeddah (KSA) was seronegative. One out of six (16.7%) RAR patients who underwent allograft transplant in the Philippines and 1/3 (33.3%) of patients who received an allograft in the USA were seropostive. None of those who underwent allograft transplant in Pakistan (n=30), Egypt (n=3) or India (n=1) were seropositive.

RAR patients who underwent allograft transplant in the city of Riyadh (8/16) or the Philippines (3/6) showed a 50% genopositivity rate. The single RAR patient who received the allograft in Jeddah was genonegative and the single RAR patient who received the allograft in India was genopositive. One out of three (33.3%) RAR patients who received an allograft in Egypt or the USA were HHV-8 genopositive compared to 9/30 (30%) of patients who received an allograft in Pakistan.

3.3.3.7 Nationality of donor

Anti-HHV-8 IgG was detected in 2/13 (15.4%) of RAR patients receiving allografts from Saudi donors; 1/6 (16.7%) from Philipino donors; 1/3 (33.3%) from American donors; and none in RAR patients with donors from Pakistan (n=30), India (n=1), Egypt (n=2) or Sudan (n=2), or those whose origin was unknown (n=4).

HHV-8 DNA was detected in 8/12 (66.7%) of RAR patients receiving allografts from Saudi donors; 9/30 (30%) from Pakistani donors; 1/1 (100%) from Indian donors; 3/6 (50%) from Philipino donors; 1/2 (50%) from Egyptian donors; 1/3 (33.3%) from American donors; and none in RAR patients with donors from Sudan (n=2), or those whose origin was unknown (n=4).

3.3.3.8 Allograft donor status

Patients receiving an allograft from a cadaver donor showed a 1/7 (14.3%) seropositivity rate compared to 2/14 (14.3%) for those receiving an allograft from a live related donor and 1/40 (2.5%) from a live unrelated donor.

Patients receiving an allograft from a cadaver donor showed a 1/7 (14.3%) genopositivity rate compared to 8/13 (61.5%) for those receiving an allograft from a live related donor and14/40 (35%) from a live unrelated donor.

3.3.3.9 Time elapsed since transplant

Anti-HHV-8 IgG was detected in 2/33 (6.1%) of RAR patients who had received the allograft within the past 2 years compared to 2/28 (7.1%) of RAR patients who had received the allograft two or more years ago ($P = 1.000$, Fisher's exact test).

HHV-8 DNA was detected in 13/33 (39.4%) of RAR patients who had received the allograft within the past 2 years compared to 10/27 (37%) of RAR patients who had received the allograft two or more years ago ($P = 1.000$, Fisher's exact test).

3.3.3.10 Hepatitis B serostatus

None of the four known hepatitis-B-positive BD or four known hepatitis-B-positive (HBsAg) CRF patients were found to be HHV-8 sero- or geno-positive. One of the two known hepatitis-B-seropositive RAR patients was found to be HHV-8 genopositive but not sero-positive.

3.3.3.11 Hepatitis C serostatus

None of the two known hepatitis-C-seropositive BD were found to be HHV-8 sero- or geno-positive. Out of the known hepatitis-C-positive CRF patients, 1/11 (9.1%) was

found to be HHV-8 seropositive compared to 11/61 (18%) of hepatitis-C-negative CRF patients (P = 0.677, Fisher's exact test). None of the 6 hepatitis-C-seropositive RAR patients were found to be HHV-8 seropositive while 4/55 (7.3%) of hepatitis-C-negative RAR patients were seropositive (P = 1.000, Fisher's exact test).

Out of 10 hepatitis-C-seropositive CRF patients, 3 (30%) were HHV-8 genopositive compared to 26/59 (44.1%) of hepatitis-C-seronegative patients (P = 0.502, Fisher's exact test). Four of 6 hepatitis-C-seropositive RAR patients (66.7%) were HHV-8 genopositive compared to 19/54 (35.2%) of hepatitis-C-seronegative patients (P = 0.191, Fisher's exact test).

3.3.3.12 Systemic drug therapy

No CRF patients were administered ciclosporin or tacrolimus. Out of 3 CRF patients receiving low doses of prednisolone, 2 (66.7%) were HHV-8 genopositive. The single CRF patient receiving a high dose of prednisolone and another receiving a high dose of mycophenolate mofetil (MMF) were HHV-8 genonegative. However, all CRF patients receiving prednisolone or MMF were seronegative.

At the time of sampling, all RAR patients were being administered a therapeutic regimen consisting of: prednisolone and ciclosporin (n=51), or prednisolone and tacrolimus (n=9), or prednisolone alone (n=1). Some patients were additionally administered a low (n=4) or high (n=26) dose of MMF. The administered dosage of each of these drugs was carefully adjusted by their attending physician as necessitated by their clinical status.

Anti-HHV-8 IgG was detected in the single RAR patient receiving prednisolone alone. Anti-HHV-8 IgG was detected in 3/51 (5.9%) of RAR patients receiving a prednisolone and ciclosporin regimen compared to none of 9 patients receiving a

prednisolone and tacrolimus regimen (P = 1.0, Fisher's exact test).

Anti-HHV-8 IgG was detected in 3/50 (6%) of RAR patients receiving high doses of ciclosporin compared to 1/10 (10%) of patients not receiving ciclosporin (P = 0.1, Fisher's exact test). Anti-HHV-8 IgG was detected in 2/44 (4.5%) of patients receiving a low dose and in 2/17 (11.8%) of those receiving a high dose of prednisolone (P = 0.308, Fisher's exact test). Anti-HHV-8 IgG was not detected in any RAR patient administered a low (n=7) or high (n=2) dose of tacrolimus. Anti-HHV-8 IgG was detected in 1/4 (25%) of RAR patients administered a low dose MMF, none of 26 patients administered a high-dose MMF (total 1/30; 3.3%), compared to 3/31 (9.7%) of RAR patients not administered MMF (P = 0.612, Fisher's exact test). No HHV-8 DNA or Anti-HHV-8 IgG was detected in the three RAR patients receiving antiviral therapy (valaciclovir).

HHV-8 DNA was detected in the single patient receiving prednisolone alone. HHV-8 DNA was detected in 22/51 (43%) of patients receiving a prednisolone and ciclosporin regimen compared to 1/9 (11.1%) of those receiving a prednisolone and tacrolimus regimen (P = 0.134, Fisher's exact test).

HHV-8 DNA was detected in the single RAR patient receiving a low dose of ciclosporin and in 21/50 (42%) of patients receiving high doses of ciclosporin (total 22/51; 43.1% administered ciclosporin) compared to 1/9 (11.1%) of patients not receiving ciclosporin (P = 0.134, Fisher's exact test). HHV-8 DNA was detected in 17/43 (39.5%) of RAR patients receiving a low dose of prednisolone and in 6/17 (35.3%) of those receiving a high dose of prednisolone (P =1.000, Fisher's exact test). HHV-8 was detected in 1/7 (14.3%) of RAR patients administered a low dose of tacrolimus and in none of 2 patients administered a high dose of tacrolimus. HHV-8 was detected in 25% (1/4) of RAR patients administered a low dose of MMF, 50%

(13/26) administered a high dose of MMF (total 14/30; 46.7% administered MMF), compared to 30% (9/30) of RAR patients not administered the MMF (P = 0.288, Fisher's exact test).

The results based on the logistic regression analysis revealed that there was no statistical evidence to suggest that any of the general characteristics (age, gender, area of origin, medication, HBV infection, HCV infection, dental Indices, relation of donor, location of transplant, months since transplant, type of renal allograft, duration of dialysis and previous transplant) increased the likelihood of the patients having detectable HHV-8 in either blood or saliva, or being anti-HHV-8-seropositive.

Table 3.8 Demographic and virological factors vs HHV-8 detection in Saudi blood donors

Characteristic		Genopositivity		Seropositivity	
		+ve No. (%)	-ve No. (%)	+ve No. (%)	-ve No. (%)
Gender					
	Female	0/2	2/2 (100)	0/2	2/2 (100)
	Male	9/176 (5.1)	167/176 (94.9)	1/176 (0.6)	175/176 (99.4)
Age (years)					
	18-20	2/12 (16.7)	10/12 (83.3)	0/12	12/12 (100)
	21-30	4/86 (4.7)	82/86 (95.3)	0/86	86/86 (100)
	31-40	3/62 (4.8)	59/62 (95.2)	1/62 (1.6)	61/62 (98.4)
	41-50	0/16	16/16 (100)	0/16	16/16 (100)
	51-60	0/2	2/2 (100)	0/2	2/2 (100)
Hepatitis B					
	Positive	0/4	4/4 (100)	0/4	4/4 (100)
	Negative	9/174 (5.2)	165/174 (94.8)	1/174 (0.6)	173/174 (99.4)
Hepatitis C					
	Positive	0/2	2/2 (100)	0/2	2/2 (100)
	Negative	9/176 (5.1)	167/176 (94.9)	1/176 (0.6)	175/176 (99.4)
Blood group					
	A	2/47 (4.3)	45/47 (95.7)	0/47	47/47 (100)
	B	1/28 (3.6)	27/28 (96.4)	0/28	28/28 (100)
	AB	0/6	6/6 (100)	0/6	6/6 (100)
	O	6/97 (6.2)	91/97 (93.8)	1/97 (1.1)	96/97 (98.9)
Total		9/178 (5.1)	169/178 (94.9)	1/178 (0.6)	177/178 (99.4)

Table 3.9 Demographic and virological factors vs HHV-8 detection in Saudi blood donors and pregnant women combined

Characteristic		Genopositivity		Seropositivity	
		+ve No. (%)	-ve No. (%)	+ve No. (%)	-ve No. (%)
Gender					
	Female	0/62	62/62 (100)	0/62	62/62 (100)
	Male	9/176 (5.1)	167/176 (94.9)	1/176 (0.6)	175/176 (99.4)
Age (years)					
	17-20	2/17 (11.8)	15/17 (88.2)	0/17	17/17 (100)
	21-30	4/119 (3.4)	115/119 (96.6)	0/119	119/119 (100)
	31-40	3/80 (3.8)	77/80 (96.2)	1/80 (1.3)	79/80 (98.7)
	41-50	0/20	20/20 (100)	0/20	20/20 (100)
	51-60	0/2	2/2 (100)	0/2	2/2 (100)
Hepatitis B					
	Positive	0/4	4/4 (100)	0/4	4/4 (100)
	Negative	9/234 (3.9)	225/234 (96.1)	1/234 (0.4)	233/234 (99.6)
Total		9/238 (3.8)	229/238 (96.2)	1/238 (0.4)	237/238 (99.6)

Table 3.10 Demographic, clinical and virological factors vs HHV-8 seroprevalence

Characteristic		CRF		RAR	
		+ve No. (%)	-ve No. (%)	+ve No. (%)	-ve No. (%)
Gender					
	Female	7/29 (24.1)	22/29 (75.9)	2/24 (8.3)	22/24 (91.7)
	Male	5/43 (11.6)	38/43 (88.4)	2/37 (5.4)	35/37 (94.6)
Age (years)					
	18-20	-	-	0/3	3/3 (100)
	21-30	0/2	2/2 (100)	0/13	13/13 (100)
	31-40	0/9	9/9 (100)	0/14	14/14 (100)
	41-50	0/11	11/11 (100)	1/20 (5)	19/20 (95)
	51-60	4/18 (22.2)	14/18 (77.8)	2/6 (33.3)	4/6 (66.7)
	61-70	5/21 (23.8)	16/21 (76.2)	1/4 (25)	3/4 (75)
	71-80	3/9 (33.3)	6/9 (66.7)	0/1	1/1 (100)
	81-90	0/2	2/2 (100)	-	-
Area of origin					
	Riyadh	4/34 (11.8)	30/34 (88.2)	0/17	17/17 (100)
	Makkah	1/7 (14.3)	6/7 (85.7)	0/3	3/3 (100)
	Madinah	-	-	0/2	2/2 (100)
	Qaseem	1/6 (16.7)	5/6 (83.3)	0/10	10/10 (100)
	E.P.	1/1 (100)	0/1	0/3	3/3 (100)
	Aseer	3/9 (33.3)	6/9 (66.7)	1/7 (14.3)	6/7 (85.7)
	Tabouk	0/1	1/1 (100)	-	-
	Hail	1/5 (20)	4/5(80)	0/1	1/1 (100)
	Jizan	1/6 (16.7)	5/6 (83.3)	0/6	6/6 (100)
	Najran	-	-	1/3 (33.3)	2/3 (66.7)
	AlBaha	0/1	1/1 (100)	1/4 (25)	3/4 (75)
	Aljouf	0/1	1/1 (100)	1/2 (50)	1/2 (50)
	Other*	0/1	1/1 (100)	0/3	3/3 (100)
Duration of dialysis					
	≤5years	4/36 (11.1)	32/36 (88.9)		
	>5years	8/36 (22.2)	28/36 (77.8)		
Previous renal transplant(s)					
	yes	0/8	8/8 (100)	0/5	5/5 (100)
	no	12/64 (18.8)	52/64 (81.2)	4/56 (7.1)	52/56 (92.9)
Location of transplant					
	KSA/Riyadh			2/17 (11.8)	15/17 (88.2)
	KSA/Jeddah			0/1	1/1 (100)
	Philippines			1/6 (16.7)	5/6 (83.3)
	Pakistan			0/30	30/30 (100)
	USA			1/3 (33.3)	2/3 (66.7)
	Egypt			0/3	3/3 (100)
	India			0/1	1/1 (100)
Nationality of donor					
	Saudi			2/13 (15.4)	11/13 (84.6)
	Pakistani			0/30	30/30 (100)
	Indian			0/1	1/1 (100)
	Philipino			1/6 (16.7)	5/6 (83.3)
	Egyptian			0/2	2/2 (100)
	American			1/3 (33.3)	2/3 (66.7)
	Sudanese			0/2	2/2 (100)
	Unknown			0/4	4/4 (100)

Table 3.10 Demographic, clinical and virological factors vs HHV-8 seroprevalence... *cont'd*

Characteristic		CRF		RAR	
		+ve No. (%)	-ve No. (%)	+ve No. (%)	-ve No. (%)
Allograft Donor status					
	Cadaver			1/7 (14.3)	6/7 (85.7)
	Live-related			2/14 (14.3)	12/14 (85.7)
	Live-unrelated			1/40 (2.5)	39/40 (97.5)
Time since transplant					
	<2 years			2/33 (6.1)	31/33 (93.9)
	≥2 years			2/28 (7.1)	26/28 (92.9)
Hepatitis B					
	positive	0/4	4/4 (100)	0/2	2/2 (100)
	negative	12/68 (17.6)	56/68 (82.4)	4/59 (6.8)	55/59 (93.2)
Hepatitis C					
	positive	1/11 (9.1)	10/11(90.9)	0/6	6/6 (100)
	negative	11/61 (18)	50/61 (82)	4/55 (7.3)	51/55 (92.7)
Ciclosporin					
	not administered	12/72 (16.7)	60/72 (83.3)	1/10 (10)	9/10 (90)
	low dose (≤75 mg OD)			0/1	1/1 (100)
	high dose (>75 mg OD)			3/50 (6)	47/50 (94)
Prednisolone					
	not administered	12/68 (17.6)	56/68 (82.4)		
	low dose (≤10 mg OD)	0/3	3/3 (100)	2/44 (4.5)	42/44 (95.5)
	high dose (>10 mg OD)	0/1	1/1 (100)	2/17 (11.8)	15/17 (88.2)
Tacrolimus					
	not administered	12/72 (16.7)	60/72 (83.3)	4/52 (7.7)	48/52 (92.3)
	low dose (≤10 mg OD)			0/7	7/7 (100)
	high dose (>10 mg OD)			0/2	2/2 (100)
MMF					
	not administered	12/71 (16.9)	59/71 (83.1)	3/31 (9.7)	28/31 (90.3)
	low dose (<1 g OD)			1/4 (25)	3/4 (75)
	high dose (≥1 g OD)	0/1	1/1 (100)	0/26	26/26 (100)
Valaciclovir					
	not administered	12/72 (16.7)	60/72 (83.3)	4/58 (6.9)	54/58 (93.1)
	administered			0/3	3/3(100)
Total		**12/72 (16.7)**	**60/72 (83.3)**	**4/61 (6.6)**	**57/61 (93.4)**

CRF, patients with chronic renal failure; RAR, renal allograft recipients; E.P, Eastern Province; *, born outside the Kingdom of Saudi Arabia: OD, once daily

Table 3.11 Demographic, clinical and virological factors vs HHV-8 genoprevalence

Characteristic		CRF		RAR	
		+ve No. (%)	-ve No. (%)	+ve No. (%)	-ve No. (%)
Gender					
	Female	15/27 (55.6)	12/27 (44.4)	7/24 (29.2)	17/24 (70.8)
	Male	14/42 (33.3)	28/42 (66.7)	16/36 (44.4)	20/36 (55.6)
Age (years)					
	18-20	-	-	0/3	3/3 (100)
	21-30	2/2 (100)	0/2	8/13 (61.5)	5/13 (38.5)
	31-40	2/9 (22.2)	7/9 (77.8)	5/14 (35.7)	9/14 (64.3)
	41-50	5/11 (45.5)	6/11 (54.5)	7/20 (35)	13/20 (65)
	51-60	3/18 (16.7)	15/18 (83.3)	2/5 (40)	3/5 (60)
	61-70	11/18 (61.1)	7/18 (38.9)	1/4 (25)	3/4 (75)
	71-80	6/9 (66.7)	3/9 (33.3)	0/1	1/1 (100)
	81-90	0/2	2/2 (100)	-	-
Area of origin					
	Riyadh	14/34 (41.2)	20/34 (58.8)	8/17 (47.1)	9/17 (52.9)
	Makkah	2/7 (28.6)	5/7 (71.4)	2/3 (66.7)	1/3 (33.3)
	Madinah	-	-	1/2 (50)	1/2 (50)
	Qaseem	1/5 (20)	4/5 (80)	4/10 (40)	6/10 (60)
	Eastern province	1/1 (100)	0/1	1/3 (33.3)	2/3 (66.7)
	Aseer	6/7 (85.7)	1/7 (14.3)	0/6	6/6 (100)
	Tabouk	0/1	1/1 (100)	-	-
	Hail	4/5 (80)	1/5 (20)	0/1	1/1 (100)
	Jizan	1/6 (16.7)	5/6 (83.3)	2/6 (33.3)	4/6 (66.7)
	Najran	-	-	1/3 (33.3)	2/3 (66.7)
	AlBaha	0/1	1/1 (100)	3/4 (75)	1/4 (25)
	Aljouf	0/1	1/1 (100)	1/2 (50)	1/2 (50)
	Other*	0/1	1/1 (100)	0/3	3/3 (100)
Duration of dialysis					
	≤5years	14/35 (40)	21/35 (60)		
	>5years	15/34 (44.1)	19/34 (55.9)		
Previous renal transplant(s)					
	yes	3/8 (37.5)	5/8 (62.5)	1/5 (20)	4/5 (80)
	no	26/61 (42.6)	35/61 (57.4)	22/55 (40)	33/55 (60)
Location of transplant					
	KSA/Riyadh			8/16 (50)	8/16 (50)
	KSA/Jeddah			0/1	1/1 (100)
	Philippines			3/6 (50)	3/6 (50)
	Pakistan			9/30 (30)	21/30 (70)
	USA			1/3 (33.3)	2/3 (66.7)
	Egypt			1/3 (33.3)	2/3 (66.7)
	India			1/1 (100)	0/1
Nationality of donor					
	Saudi			8/12 (66.7)	4/12 (33.3)
	Pakistani			9/30 (30)	21/30 (70)
	Indian			1/1 (100)	0/1
	Philipino			3/6 (50)	3/6 (50)
	Egyptian			1/2 (50)	1/2 (50)
	American			1/3 (33.3)	2/3 (66.7)
	Sudanese			0/2	2/2 (100)
	Unknown			0/4	4/4 (100)

Table 3.11 Demographic, clinical and virological factors vs HHV-8 genoprevalence... *cont'd*

Characteristic		CRF		RAR	
		+ve No. (%)	-ve No. (%)	+ve No. (%)	-ve No. (%)
Allograft Donor status					
	Cadaver			1/7 (14.3)	6/7 (85.7)
	Live-related			8/13 (61.5)	5/13 (38.5)
	Live-unrelated			14/40 (35)	26/40 (65)
Time since transplant					
	<2 years			13/33 (39.4)	20/33 (60.6)
	≥2 years			10/27 (37)	17/27 (63)
Hepatitis B					
	positive	0/4	4/4 (100)	1/2 (50)	1/2 (50)
	negative	29/65 (44.6)	36/65 (55.4)	22/58 (37.9)	36/58 (62.1)
Hepatitis C					
	positive	3/10 (30)	7/10 (70)	4/6 (66.7)	2/6 (33.3)
	negative	26/59 (44.1)	33/59 (55.9)	19/54 (35.2)	35/54 (64.8)
Ciclosporin					
	not administered	29/69 (42)	40/69 (58)	1/9 (11.1)	8/9 (88.9)
	low dose (≤75 mg OD)			1/1 (100)	0/1
	high dose (>75 mg OD)			21/50 (42)	29/50 (58)
Prednisolone					
	not administered	27/65 (41.5)	38/65 (58.5)		
	low dose (≤10 mg OD)	2/3 (66.7)	1/3 (33.3)	17/43 (39.5)	26/43 (60.5)
	high dose (>10 mg OD)	0/1	1/1 (100)	6/17 (35.3)	11/17 (64.7)
Tacrolimus					
	not administered	29/69 (42)	40/69 (58)	22/51 (43.1)	29/51 (56.9)
	low dose (≤10 mg OD)			1/7 (14.3)	6/7 (85.7)
	high dose (>10 mg OD)			0/2	2/2 (100)
MMF					
	not administered	29/68 (42.6)	39/68 (57.4)	9/30 (30)	21/30 (70)
	low dose (<1 g OD)			1/4 (25)	3/4 (75)
	high dose (≥1 g OD)	0/1	1/1 (100)	13/26 (50)	13/26 (50)
Valaciclovir					
	not administered	29/69 (42)	40/69 (58)	23/57 (40.4)	34/57 (59.6)
	administered			0/3	3/3 (100)
Total		**29/69 (42)**	**40/69 (58)**	**23/60 (38.3)**	**37/60 (61.7)**

CRF, patients with chronic renal failure; RAR, renal allograft recipients; E.P, Eastern Province; *, born outside the Kingdom of Saudi Arabia; OD, once daily

3.3.4 Whole saliva supernate viral load

The HHV-8 viral load of WMS^s, showing positive results for both K1/V1 and KS330 DNA, ranged between 8.6×10^3 and 12×10^7 genome-copies/ml (Table 3.12). HHV-8 was detected in PE samples from patients only when their WMS^s viral load exceeded 80,000 genome-copies/ml and from BE samples only when their WMS^s viral load exceeded 200,000 genome-copies/ml. The load in WMS^s obtained from CRF24, in whom HHV-8 DNA was also found in both BE and PE, was particularly high.

Table 3.12 WMS[s] HHV-8 viral loads compared to sample genopositivity

CRF ID number	CD19		CD31		CD45		WMS[s]		WMS[c]		PS		BE		PE		HHV-8 viral load WMS/PS (copies/ml)
	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	
10	neg	neg	neg	neg	neg	neg	pos	pos	pos	pos	NA	NA	neg	neg	neg	pos	151500
23	neg	neg	neg	pos	pos	pos	pos	pos	pos	pos	NA	NA	neg	neg	neg	neg	8641
24	pos	pos	neg	pos	pos	pos	pos	pos	pos	pos	NA	NA	pos	pos	pos	pos	119562500
56	neg	neg	neg	neg	neg	neg	pos	pos	pos	pos	neg	neg	neg	pos	pos	neg	241050
57	pos	neg	neg	neg	pos	pos	pos	pos	pos	pos	neg	neg	neg	neg	neg	pos	83225

CRF, patients with chronic renal failure; WMS[s], whole-mouth saliva supernate fraction; WMS[c], whole-mouth saliva cellular fraction; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; NA, sample not available

3.3.5 Association between oral inflammation and salivary HHV-8 viral load

No association was observed between salivary viral load and oral inflammation, as indicated by the plaque, gingival and bleeding oral indices ($P > 0.05$, Spearman's rank correlation coefficient) (Table 3.13).

Table 3.13 Patient oral health in relation to HHV-8 WMS^{s} viral loads

Sample	WMS^{s} viral load copies/ml	Plaque index	Gingival index	Bleeding Index
CRF10*	151,500	2.25	-	-
CRF23	8,641	1.88	1.25	0.50
CRF24	119,562,500	1.04	0	0
CRF56	241,050	1.06	0.63	0
CRF57	83,225	1.42	1.54	1.08

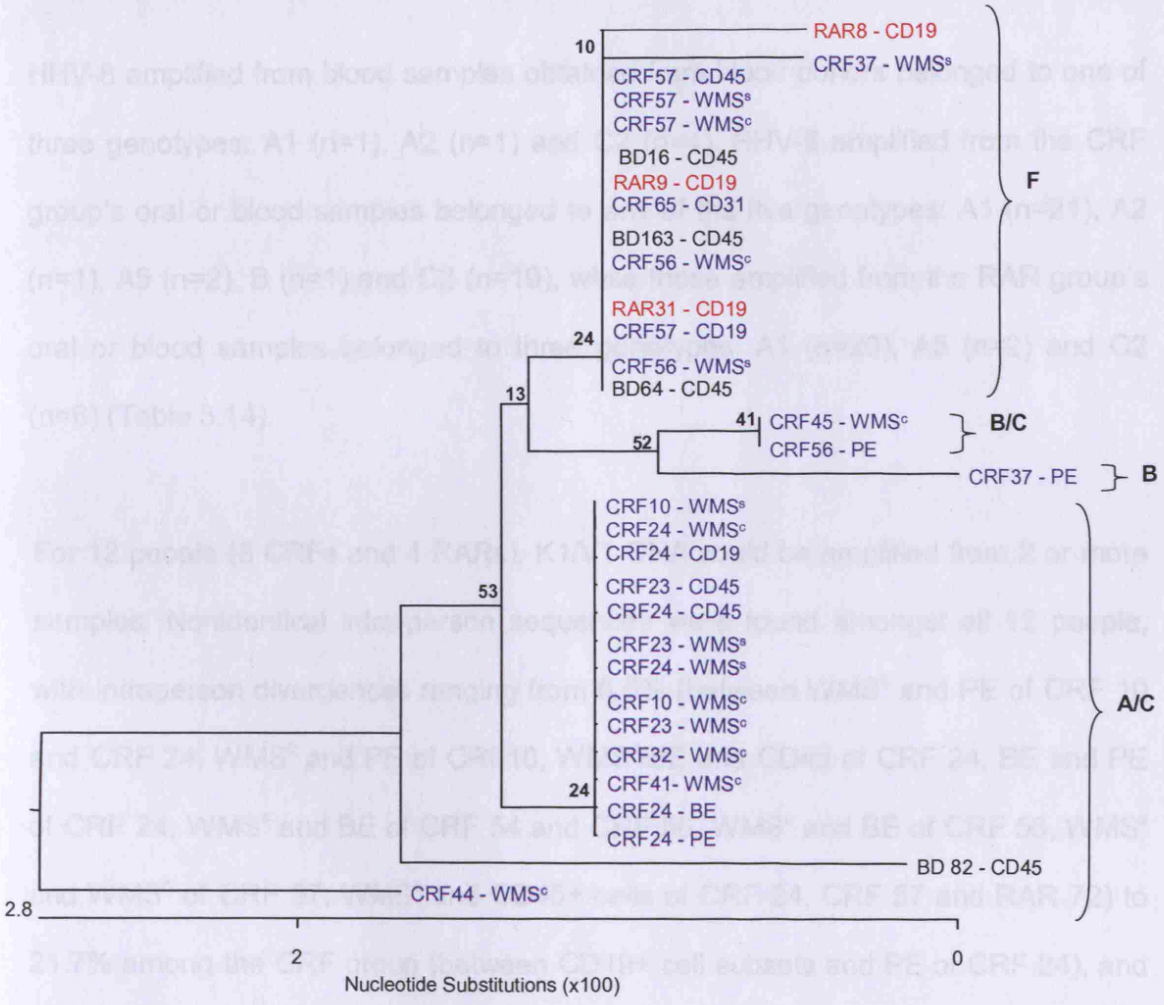
* Examination during dialysis session; hence no gingival or bleeding index obtained.
CRF, patients with chronic renal failure

3.3.6 Sequence diversity

3.3.6.1 KS330

A dendrogram displaying the diversity of consensus KS330 sequences among the samples, and their genotypic assignments is shown in Fig 3.4. The KS330 genotype of blood and oral samples obtained from the three groups that showed positive results belonged to four different subgroups (A/C, B/C, B and F), with a maximum divergence of 4.3%. All positive blood samples of renal allograft recipients and the majority of blood donors (3/4) belonged to the F genotype. HHV-8 amplified from the blood samples of the CRF group belonged to either A/C or F genotype, while those amplified from the oral samples of this same group belonged to any one of the four genotypes (Fig 3.4, Tables 3.15, 3.16 and 3.17). Amongst the six CRF patients with multiple KS330-positive samples, four (given patient assignments CRFs 10, 23, 24 and 57) had identical intra-person KS330 sequences. Intra-person sequence diversities of up to 1.2% were detected between CRF 56's saliva samples and PE, and up to 1.8% between CRF 37's WMS^s and PE. The genotype assignments of saliva and PE samples from these two CRF patients were consequently different: genotype F for the sequences obtained from saliva and genotypes B/C for PE obtained from CRF56 and B from that obtained from CRF37.

Figure 3.4 Diversity of consensus KS330 sequences. Bootstrapping for 1000 replicates is noted as a percentage at major branch points.



3.3.6.2 K1/V1

A dendrogram displaying the diversity of BD, CRF and RAR consensus K1/V1 sequences, and their genotypic assignments is shown in Fig 3.5. The K1/V1 genotypes obtained from these three groups blood and oral samples belonged to five different subgroups (A1, A2, A5, B and C2) with a maximum divergence of 28.3%.

HHV-8 amplified from blood samples obtained from blood donors belonged to one of three genotypes: A1 (n=1), A2 (n=1) and C2 (n=4). HHV-8 amplified from the CRF group's oral or blood samples belonged to any of the five genotypes: A1 (n=21), A2 (n=1), A5 (n=2), B (n=1) and C2 (n=19), while those amplified from the RAR group's oral or blood samples belonged to three genotypes: A1 (n=20), A5 (n=2) and C2 (n=6) (Table 3.14).

For 12 people (8 CRFs and 4 RARs), K1/V1 DNA could be amplified from 2 or more samples. Nonidentical intra-person sequences were found amongst all 12 people, with intraperson divergences ranging from 0.5% (between WMS^s and PE of CRF 10 and CRF 24, WMS^c and PE of CRF10, WMS^c/BE and CD45 of CRF 24, BE and PE of CRF 24, WMS^s and BE of CRF 54 and CRF 56, WMS^c and BE of CRF 56, WMS^s and WMS^c of CRF 57, WMS^s and CD45+ cells of CRF 24, CRF 57 and RAR 72) to 21.7% among the CRF group (between CD19+ cell subsets and PE of CRF 24), and 21.1% among the RAR group (between CD45+ cell subsets and PE of RAR 10).

Amongst the 8 CRF patients with multiple K1/V1 positive samples, four (CRFs 8, 23, 24 and 57) carried sequences belonging to 2 genotypes (A1 and A2, or A1 and C2). These were the CD19+ and CD31+ cells of CRF 8 (A1 and A2, respectively); CD31+ cells and CD45+ cells/saliva (WMS^s and WMS^c) of CRF 23 (A1 and C2, respectively); CD19+/CD31+ cells and CD45+ cells/saliva/BE/PE of CRF 24 (A1 and C2, respectively); and PE and saliva/CD45+ cell samples of CRF 57 (A1 and C2,

respectively).

Amongst the 4 RAR patients with multiple K1/V1-positive samples, three (RARs 10, 23 and 61) carried sequences belonging to 2 genotypes (A1 and C2, or A1 and A5). These were the CD45+ cells and BE of RAR 10 (A1 and C2, respectively); CD19+ cells and WMS^c of RAR 23 (C2 and A1, respectively); WMS^s/BE and WMS^c of RAR 61 (A1 and A5, respectively).

For the 4 CRF and 1 RAR patient whose samples were found to carry nonidentical sequences of the same genotype, intraperson/intersample sequences were >5% divergent in the case of only one patient: CRF 43 BE and PS (5.9%); WMS^s and PS (5.4%).

Identical intra-person sequences could be identified: between WMS^s and WMS^c of both CRF 10 and CRF 56, WMS^s and BE of CRF 24, and WMS^c and CD45+ cells of CRF 57, all belonging to the C2 genotype (Figure 3.5, Table 3.16).

There was no evidence that the genotype profile (Table 3.14) was different between the oral and blood samples of CRF patients (P = 0.084, Pearson Chi-Square) or between the oral and blood samples of RAR patients (P = 0.039, Pearson Chi-Square). Although the Chi square test results revealed that there was a statistically significant difference, this result should be treated with caution, as the number of cells, employed in the statistical analysis, with an expected count less than 5 were as high as 67%.

The phylogenetic tree in Figure 3.5, shows a number of CRF patients exhibiting identical K1/V1 sequences, some of whom were dialyzed during the same shift and in the same room (CRF7-CD31/CRF8-CD19 and CRF2-CD31/CRF15-BE) while others

were dialyzed during the same shift but in different rooms (CRF46-WMS^{c}/CRF48-CD31 and CRF7-CD31 & CRF8-CD19/ CRF61-WMS^{c}). In addition, sequences from a number of other CRF patients differed by 1 to 3 nucleotides and were identical at the amino acid level (CRF2-CD31 & CRF15-BE/CRF43-BE; CRF7-CD31, CRF8-CD19 & CRF61-WMS^{c}/CRF24-CD19 & CRF12-PE; CRF43-WMS^{s}/CRF57-WMS^{c}; CRF60-WMS^{c}/ CRF24- WMS^{c}), some of whom were dialyzed during the same shift and in the same room (CRF61-WMS^{c}/CRF24-CD19 & CRF12-PE), while others were dialyzed during different shifts (CRF2-CD31 & CRF15-BE/CRF43-BE; CRF43-WMS^{s}/CRF57-WMS^{c}; CRF60- WMS^{c}/ CRF24- WMS^{c}).

Possible linkages between the KS330 and K1/V1 genotypes (as determined by direct sequencing) were analyzed (Table 3.15, 3.16 and 3.17). No obvious patterns of linkage were observed.

Figure 3.5 Diversity of consensus K1/V1 sequences. Bootstrapping for 1000 replicates is noted as a percentage at major branch points.

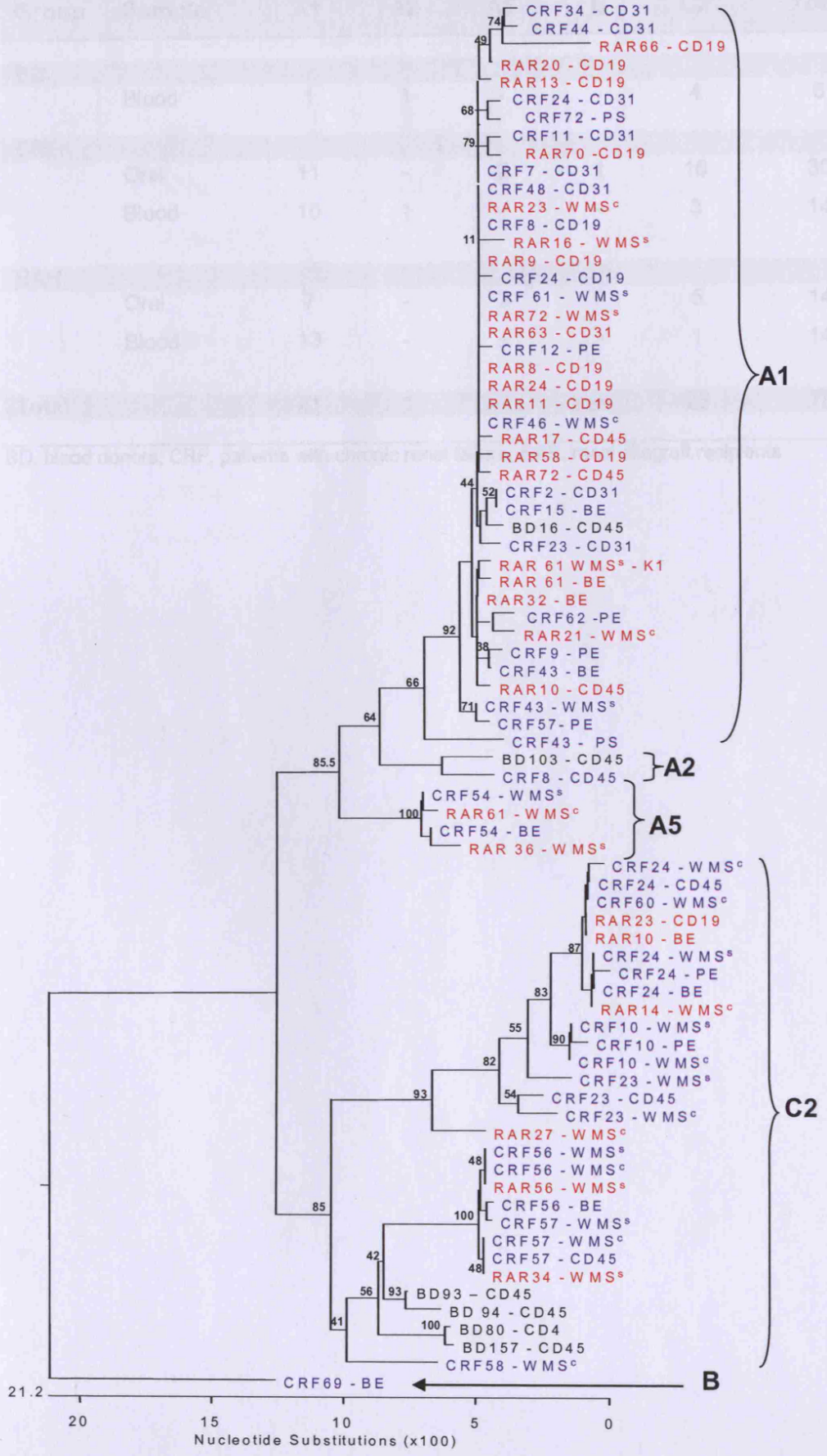


Table 3.14 K1/V1 genotype assignments according to study groups

		Genotype					
Group	**Sample**	**A1**	**A2**	**A5**	**B**	**C2**	**Total**
BD							
	Blood	1	1	-	-	4	6
CRF							
	Oral	11	-	2	1	16	30
	Blood	10	1	-	-	3	14
RAR							
	Oral	7	-	2	-	5	14
	Blood	13	-	-	-	1	14
Total		41	1	4	1	25	78

BD, blood donors; CRF, patients with chronic renal failure; RAR, renal allograft recipients

Table 3.15 Linkage analysis of KS330 and K1/V1 amongst BD CD45+ cells

Patient ID number	KS330 genotype	K1/V1 genotype
BD16	F	A1
BD64	F	-
BD82	A/C	-
BD80	-	C2
BD93	-	C2
BD94	-	C2
BD103	-	A2
BD157	-	C2
BD163	F	-

BD, blood donor

Table 3.16 Linkage analysis of KS330 and K1/V1 amongst CRF patients

Patient ID number	Patient sample	KS330 genotype	K1/V1 genotype
CRF2	CD31	-	A1
CRF7	CD31	-	A1
CRF8	CD19	-	A1
	CD45	-	A2
CRF9	PE	-	A1
CRF10	WMS^s	A/C	C2
	WMS^c	A/C	C2
	PE	-	C2
CRF11	CD31	-	A1
CRF12	PE	-	A1
CRF15	BE	-	A1
CRF23	CD31	-	A1
	CD45	A/C	C2
	WMS^s	A/C	C2
	WMS^c	A/C	C2
CRF24	CD19	A/C	A1
	CD31	-	A1
	CD45	A/C	C2
	WMS^s	A/C	C2
	WMS^c	A/C	C2
	BE	A/C	C2
	PE	A/C	C2
CRF32	WMS^c	A/C	-
CRF34	CD31	-	A1
CRF37	WMS^s	F	-
	PE	B	-
CRF41	WMS^c	A/C	-
CRF43	WMS^s	-	A1
	PS	-	A1
	BE	-	A1
CRF44	CD31	-	A1
	WMS^c	A/C	-

Table 3.16 Linkage analysis of KS330 and K1/V1 amongst CRF patients... *cont'd*

Patient ID number	Patient sample	KS330 genotype	K1/V1 genotype
CRF45	WMS^c	B/C	-
CRF46	WMS^c	-	A1
CRF48	CD31	-	A1
CRF54	WMS^s	-	A5
	BE	-	A5
CRF56	WMS^s	F	C2
	WMS^c	F	C2
	BE	-	C2
	PE	B/C	-
CRF57	CD19	F	-
	CD45	F	C2
	WMS^s	F	C2
	WMS^c	F	C2
	PE	-	A1
CRF58	WMS^c	-	C2
CRF60	WMS^c	-	C2
CRF61	WMS^s	-	A1
CRF62	PE	-	A1
CRF65	CD31	F	-
CRF69	BE	-	B
CRF72	PS	-	A1

CRF, patient with chronic renal failure; WMS^s, whole mouth saliva supernate fraction; WMS^c, whole mouth saliva cellular fraction; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; - , not detected

Table 3.17 Linkage analysis of KS330 and K1/V1 amongst RAR patients

Patient ID number	Patient sample	KS330 genotype	K1/V1 genotype
RAR8	CD19	F	A1
RAR9	CD19	F	A1
RAR10	CD45	-	A1
	BE	-	C2
RAR11	CD19	-	A1
RAR13	CD19	-	A1
RAR14	WMS^c	-	C2
RAR16	WMS^s	-	A1
RAR17	CD45	-	A1
RAR20	CD19	-	A1
RAR21	WMS^c	-	A1
RAR23	CD19	-	C2
	WMS^c	-	A1
RAR24	CD19	-	A1
RAR27	WMS^c	-	C2
RAR31	CD19	F	-
RAR32	BE	-	A1
RAR34	WMS^s	-	C2
RAR36	WMS^s	-	A5
RAR56	WMS^s	-	C2
RAR58	CD19	-	A1
RAR61	WMS^s	-	A1
	WMS^c	-	A5
	BE	-	A1
RAR63	CD31	-	A1
RAR66	CD19	-	A1

Table 3.17 Linkage analysis of KS330 and K1/V1 amongst RAR patients.. *cont'd*

Patient ID number	Patient sample	KS330 genotype	K1/V1 genotype
RAR70	CD19	-	A1
RAR72	CD45	-	A1
	WMS^s	-	A1

RAR, renal allograft recipient; WMS^s, whole mouth saliva supernate fraction; WMS^c, whole mouth saliva cellular fraction; BE, buccal exfoliate; - , not detected

3.3.7 Relation between HHV-8 K1/V1 genotype and selected variables

The majority of CRF and RAR HHV-8 genopositive patients were found to carry the A1 genotype (36/47%; 76.6), with seven patients carrying more than one genotype (Table 3.18).

All CRF and RAR genopositive patients originating from the Eastern province (n=2), Madina (n=1) and Aljouf (n=1), in addition to the majority of the patients originating from Riyadh (15/24), Aseer (4/7), Makkah (3/4), Hail (3/4), Albaha (2/3), Jizan (2/3) and Qaseem (2/3) carried the K1/V1 genotype A1, with three patients from Riyadh, two from Aseer and one from both Hail and Najran carrying two genotypes each. The two patients from Aseer, the single patient from Hail and two of the three patients from Riyadh carried both A1 and C2 genotypes, while the third patient from Riyadh carried both A1 and A2 genotypes. The single patient from Najran carried both A1 and A5 genotypes. Only one patient from Riyadh was found to carry B genotype (Figure 3.6).

Patients with A1 genotype varied in age from 21 yr to 72 yr. Patients with A5 genotype were ≤ 36 yr and those with C2 genotype ≥ 39 yr. The single patient exhibiting genotype B was 65 yr. All patients carrying more than one genotype were ≥ 53 yr, with the exception of one patient, who was 26 yr (Figure 3.7). However, no statistically significant association (P = 0.529, ANOVA) was found between K1/V1 genotype and the mean age of CRF and RAR patients.

The percentage of male patients carrying A1 genotype solely was 18/27 (66.7%) compared to 11/20 (55%) of female patients carrying A1 genotype (P = 0.546, Fisher's exact test). The percentage of male patients carrying A5 genotype solely was 1/27 (3.7%) compared to 1/20 (5%) of female patients carrying A5 genotype (P = 0.567, Fisher's exact test), while the percentage of male patients carrying C2

genotype solely was 4/27 (14.8%) compared to 4/20 (20%) of female patients carrying C2 genotype (P = 0.707, Fisher's exact test). The single patient carrying B genotype was male. The percentage of male patients carrying two HHV-8 genotypes was 3/27 (11.1%) compared to 4/20 (20%) of females (P = 0.438, Fisher's exact test) (Figure 3.8).

All amplifiable HHV-8 K1/V1 from CD31+ cells (n=9) and PS (n=2) were found to carry the A1 genotype. Additionally, A5 HHV-8 K1/V1 genotype was detected in oral samples only (WMS and BE), while detection of A2 genotype was limited to CD45+ cells. However, WMS, BE and CD45+ cells obtained from other patients could also carry another genotype (A1, B, or C2) (Table 3.19, Figure 3.9).

All three patients with a history of a previous transplant carried A1 genotype compared to 26/44 (59%) of patients without a previous transplant (P = 0.921, Pearson Chi-Square). None of the CRF or RAR patients with more than one HHV-8 K1/V1 genotype (0/7) had a history of a previous transplant compared to 3/40 (7.5%) of patients with only one HHV-8 K1/V1 genotype (P = 1, Fisher's Exact test). The mean age of the seven patients with more than one HHV-8 K1/V1 genotype was 54.6 yr, while the the mean age of the forty patients with only one genotype was 47.9 yr (P = 0.304, independent samples t -test). All three RAR patients carrying more than one genotype compared to 9/20 (45%) of RAR patients with one genotype had received the kidney allograft within the past 2 yr (P = 0.217, Fisher's Exact test). 2/3 (66.7%) of RAR patients carrying more than one genotype received the allografts from a relative compared to 7/20 (35%) of RAR patients carrying a single genotype (P= 0.538, Fisher's Exact test). However, these differences were not statistically significant. All Hep-C-positive CRF and RAR patients with positive HHV-8 K1/V1 were found to carry an A genotype (A1: 1 CRF patient and 3 RAR patients, A5: 1 RAR patient).

Table 3.18 HHV-8 K1/V1 genotypes amongst CRF and RAR patients (combined)

K1/V1 genotype	Frequency	Percent (%)
A1 only	29	61.7
A5 only	2	4.3
B only	1	2.1
C2 only	8	17.0
A1+A2	1	2.1
A1+C2	5	10.6
A1+A5	1	2.1
Total	47	100

Figure 3.6 Stacked column chart illustrating the distribution of K1/V1 genotypes in CRF and RAR patients according to area of origin

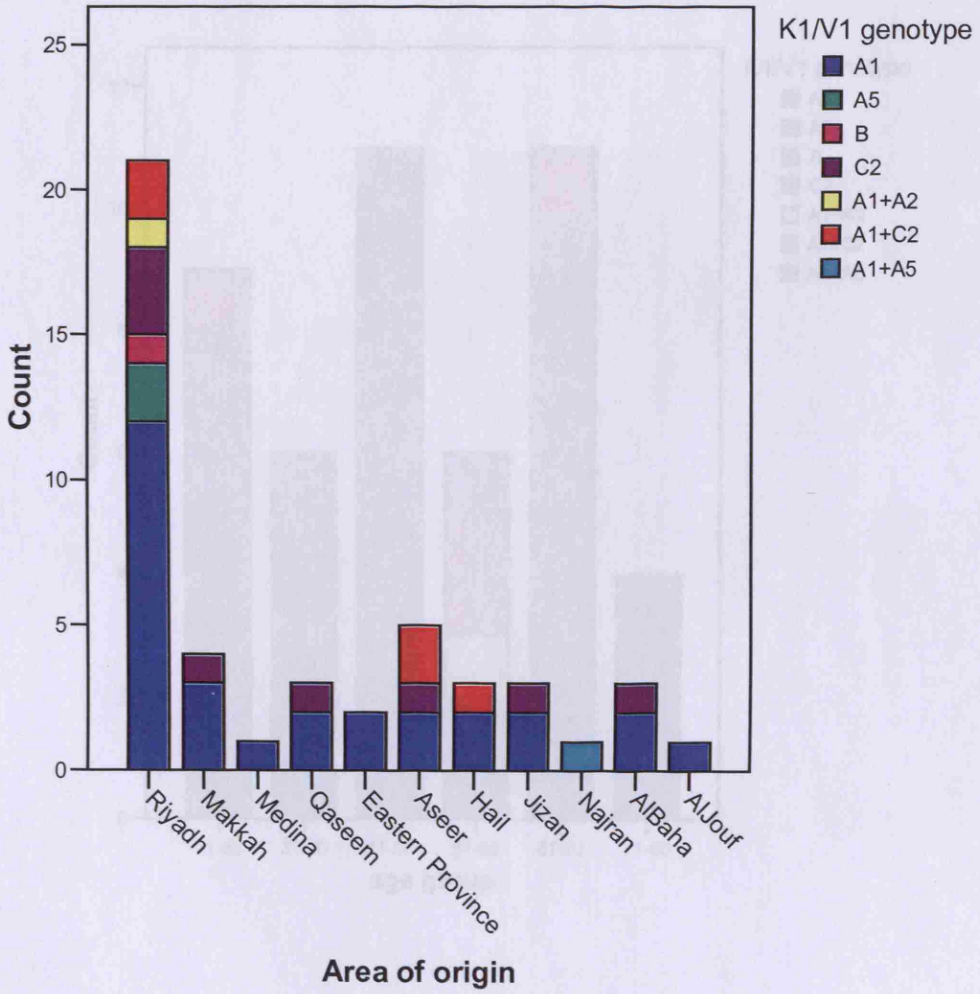


Figure 3.7 Stacked column chart illustrating the distribution of K1/V1 genotypes according to age

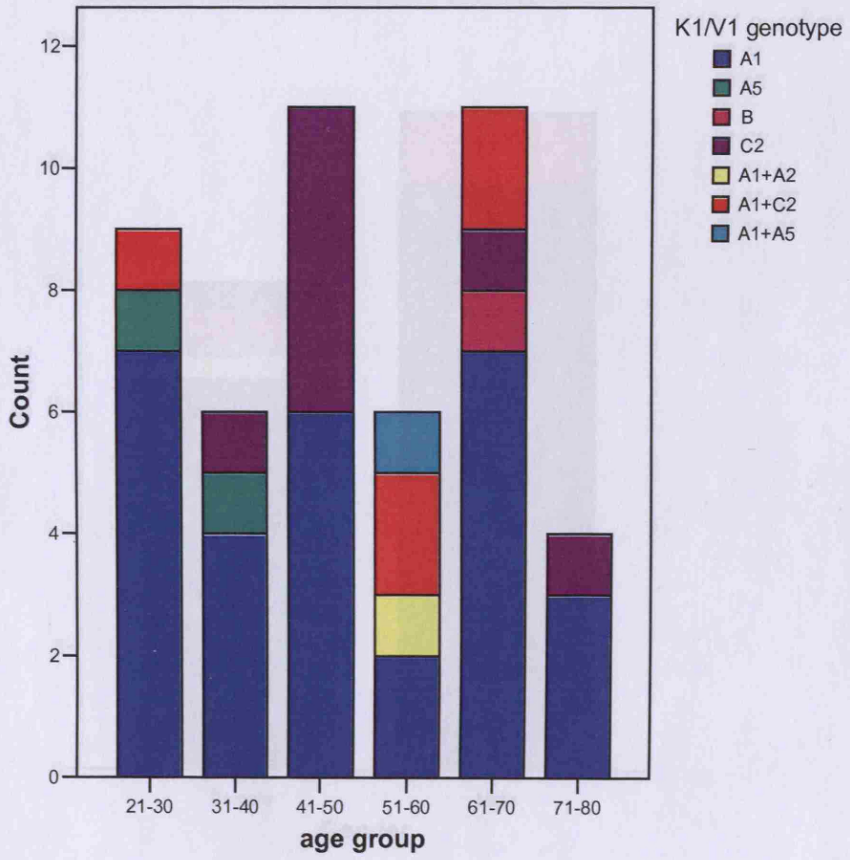


Figure 3.8 Stacked column chart illustrating the distribution of K1/V1 genotypes according to gender

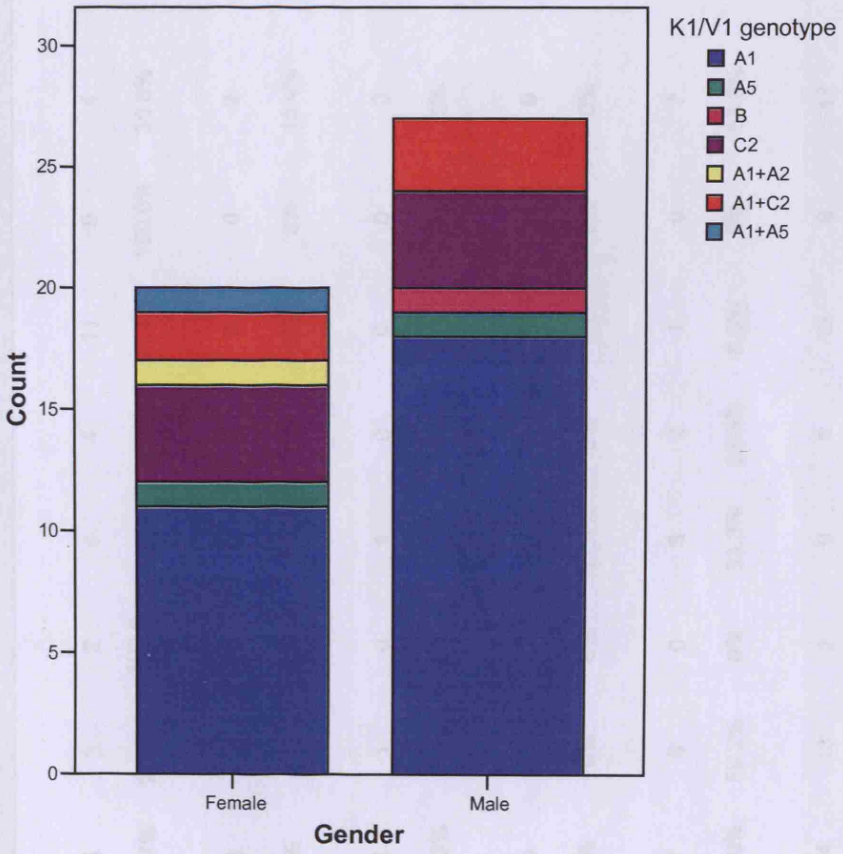
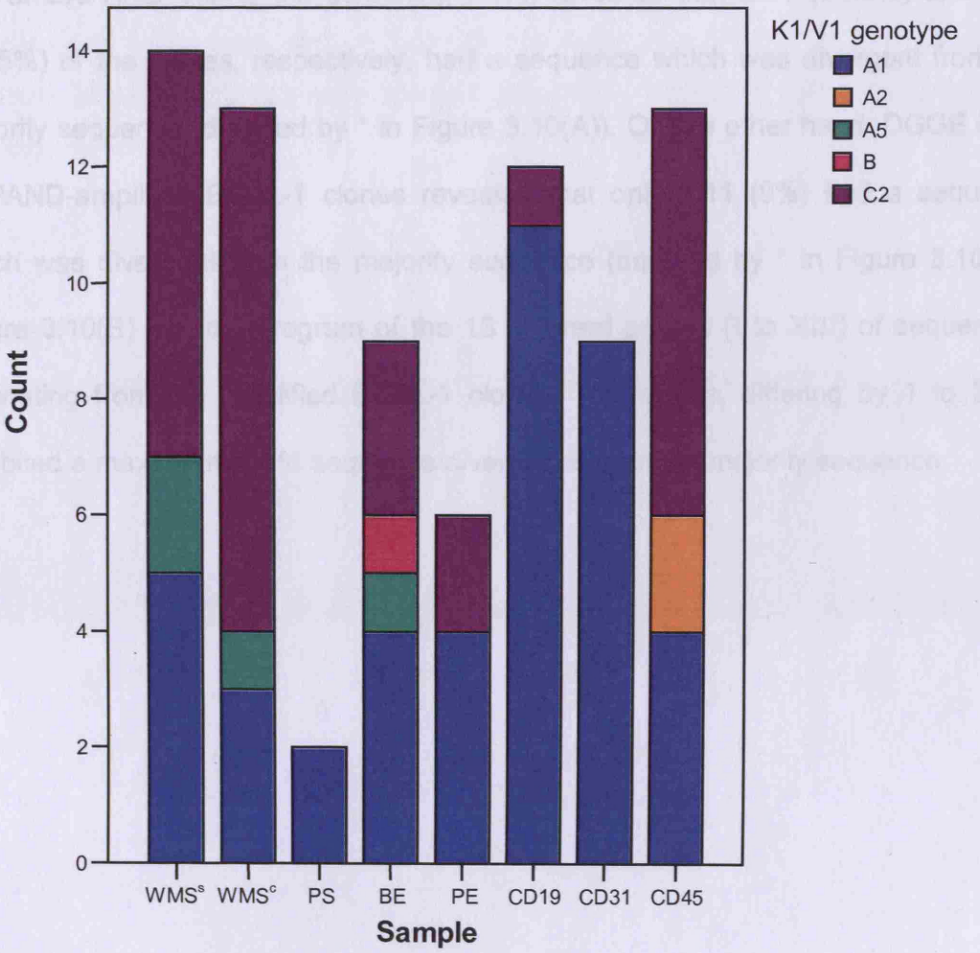


Table 3.19 Distribution of HHV-8 K1/V1 genotypes in relation to sample type

			Sample								Total
			WMS[s]	WMS[c]	PS	BE	PE	CD19	CD31	CD45	
Genotype											
	A1	count	5	3	2	4	4	11	9	4	42
		% within sample	35.7%	23.1%	100.0 %	44.4%	66.7%	91.7%	100.0%	30.8%	53.8%
	A2	count	0	0	0	0	0	0	0	2	2
		% within sample	0%	0%	0%	0%	0%	0%	0%	15.4%	2.6%
	A5	count	2	1	0	1	0	0	0	0	4
		% within sample	14.3%	7.7%	0%	11.1%	0%	0%	0%	0%	5.1%
	B	count	0	0	0	1	0	0	0	0	1
		% within sample	0%	0%	0%	11.1%	0%	0%	0%	0%	1.3%
	C2	count	7	9	0	3	2	1	0	7	29
		% within sample	50.0%	69.2%	0%	33.3%	33.3%	8.3%	0%	53.8%	37.2%
Total		count	14	13	2	9	6	12	9	13	78
		% within sample	100%	100%	100%	100%	100%	100%	100%	100%	100%

WMS[s], whole-mouth saliva supernate fraction; WMS[c], whole-mouth saliva cellular fraction; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; CD19, CD31 and CD45, PBMC subsets

Figure 3.9 Stacked column chart illustrating the distribution of K1/V1 genotypes in CRF and RAR patients according to sample type



WMS^{s}, supernate fraction of whole-mouth saliva; WMS^{c}, cellular fraction of whole-mouth saliva; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; CD19, CD31& CD45, PBMC subsets.

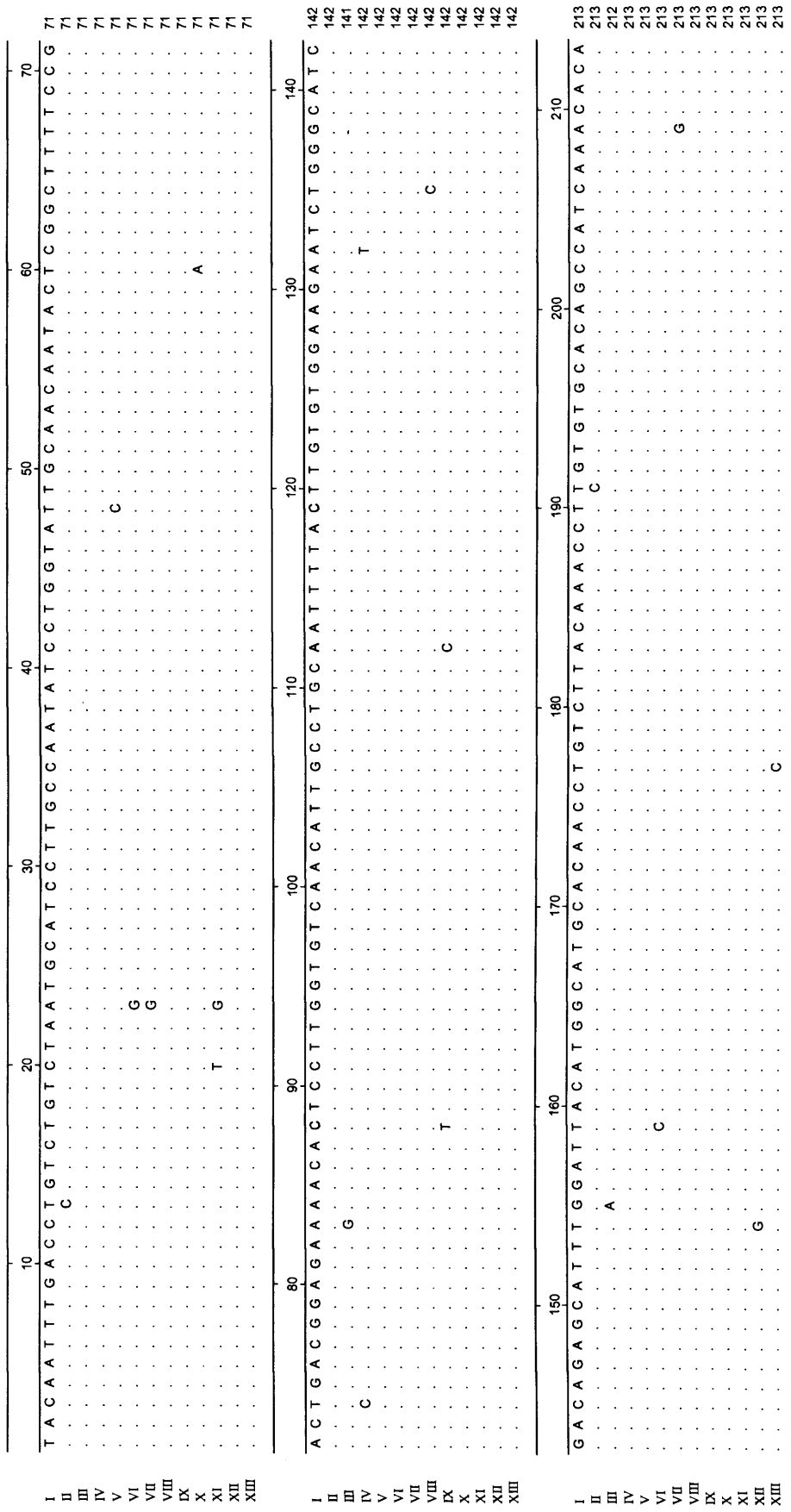
3.3.8 Extent of *Taq* polymerase-induced misincorporation

DGGE of 11 *Taq* polymerase and 11 *Taq* polymerase + EXPAND -amplified BCBL-1 clones (using *Taq* polymerase to amplify ORF K1/V1 PCR product followed by the use of EXPAND during the screening PCR) revealed that 6/11 (54.5%) and 5/11 (45.5%) of the clones, respectively, had a sequence which was divergent from the majority sequence (denoted by * in Figure 3.10(A)). On the other hand, DGGE of 11 EXPAND-amplified BCBL-1 clones revealed that only 1/11 (9%) had a sequence which was divergent from the majority sequence (denoted by * in Figure 3.10(A)). Figure 3.10(B) is a dendrogram of the 13 different groups (I to XIII) of sequences, originating from the amplified BCBL-1 clones. The clones, differing by 1 to 3 bp, exhibited a maximum of 1% sequence divergence from the majority sequence.

Figure 3.10 (A) DGGE results from PCR BCBL-1 ORF K1/V1 products generated using Taq DNA polymerase and the EXPAND system; Arabic numerals represent assigned clone numbers, coinciding with lane positions in the gel; colony products are assigned different Roman numerals. Lanes marked with a * indicate those products with a divergent sequence from the majority sequence (I), (B) A comparison of nucleotide sequences derived from the 13 different groups (I to XIII) of colony products. Dots indicate residues occupying positions aligned to those of the majority sequence (I) at top.

A)

B)



3.3.9 Intra-person K1/V1 nucleotide sequence differences as studied by DGGE

3.3.9.1 CRF 24

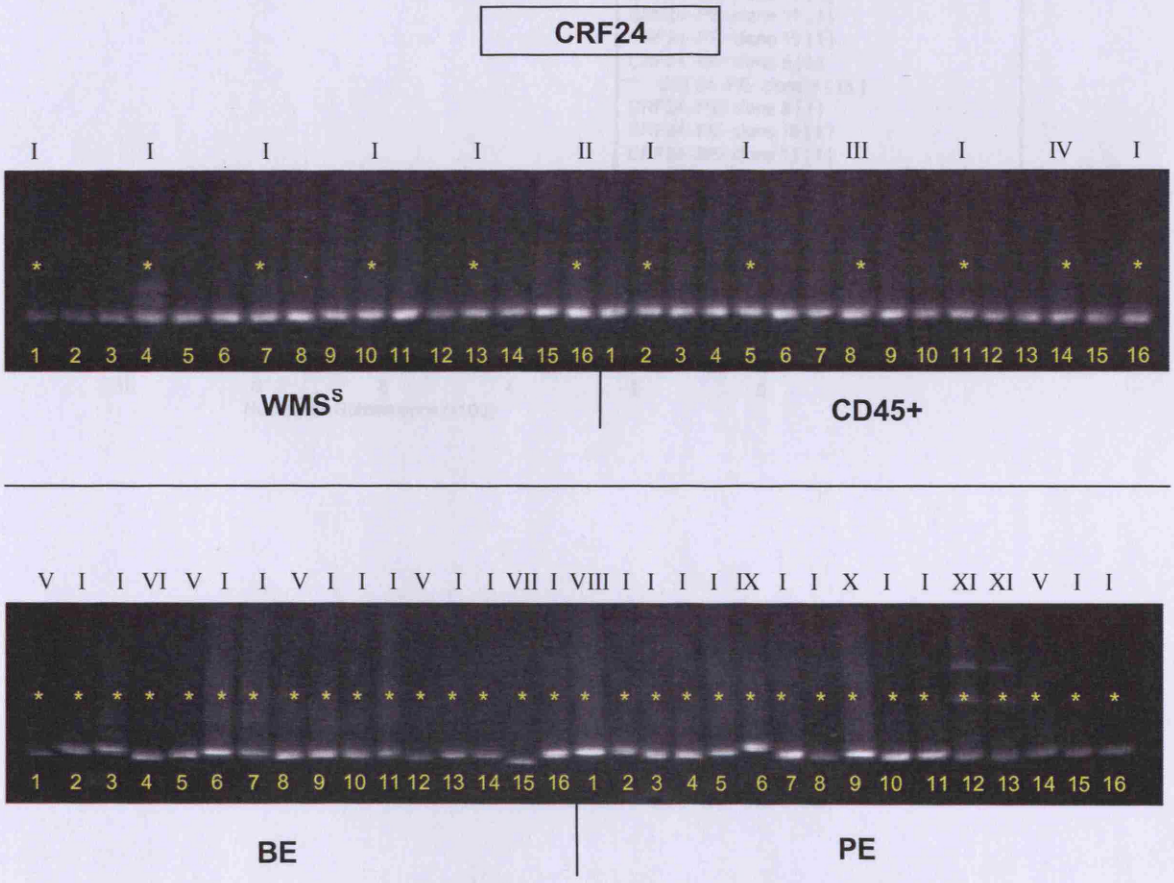
High-fidelity PCR of sample extracts from CD45+ cells, WMS^s, BE and PE of CRF 24 yielded K1/V1 DNA. Clones were generated from which differences in nucleotide sequence could be analyzed by the combined DGGE-sequencing approach. Figure 3.11 is a composite of representative DGGE gel pictures showing migration distances attained by amplicons generated from clonal inserts; each gel accommodates two samples with K1/V1 DNA (16 clones/sample). All PCR products that yielded minority bands and some products representing the majority were sequenced (denoted by * in Figure 3.11(A); Arabic numerals correspond to clone numbers). Figure 3.11(B) is a dendrogram depicting the extent of inter- and intra-sample K1/V1 nucleotide sequence diversity. A total of 11 different groups (I to XI) of sequences originating from CD45+ cells, WMS^s, BE and PE of CRF 24 were detected. A comparison of the nucleotide sequences is shown in Figure 3.11(C). The consensus ORF K1/V1 sequence from CD45+ cells was identical to the majority sequence, while the consensus ORF K1/V1 sequences from WMS^s and BE were identical to sequence group IV. The consensus ORF K1/V1 sequence from PE was not identical to any of the 11 sequence groups and was 0.9% divergent from the majority sequence.

Analysis of clones from minority sequences permitted intrasample K1/V1 variability to be further characterized from individual samples. An intra-sample sequence divergence was observed amongst the CD45+ ($\leq$ 1.4%), WMS^s ($\leq$ 0.5%), BE ($\leq$ 20.4%), PE ($\leq$ 0.9%) clones. DGGE and nucleotide sequencing analyses confirmed findings from direct K1/V1 PCR sequencing that CRF 24 carried >1 genotype. For the BE sample, direct sequencing of K1/V1 generated sequences assignable to genotype C2, but clonal analysis revealed carriage of minority sequences VI and VII generated from clones 4 and 15 which belong to another

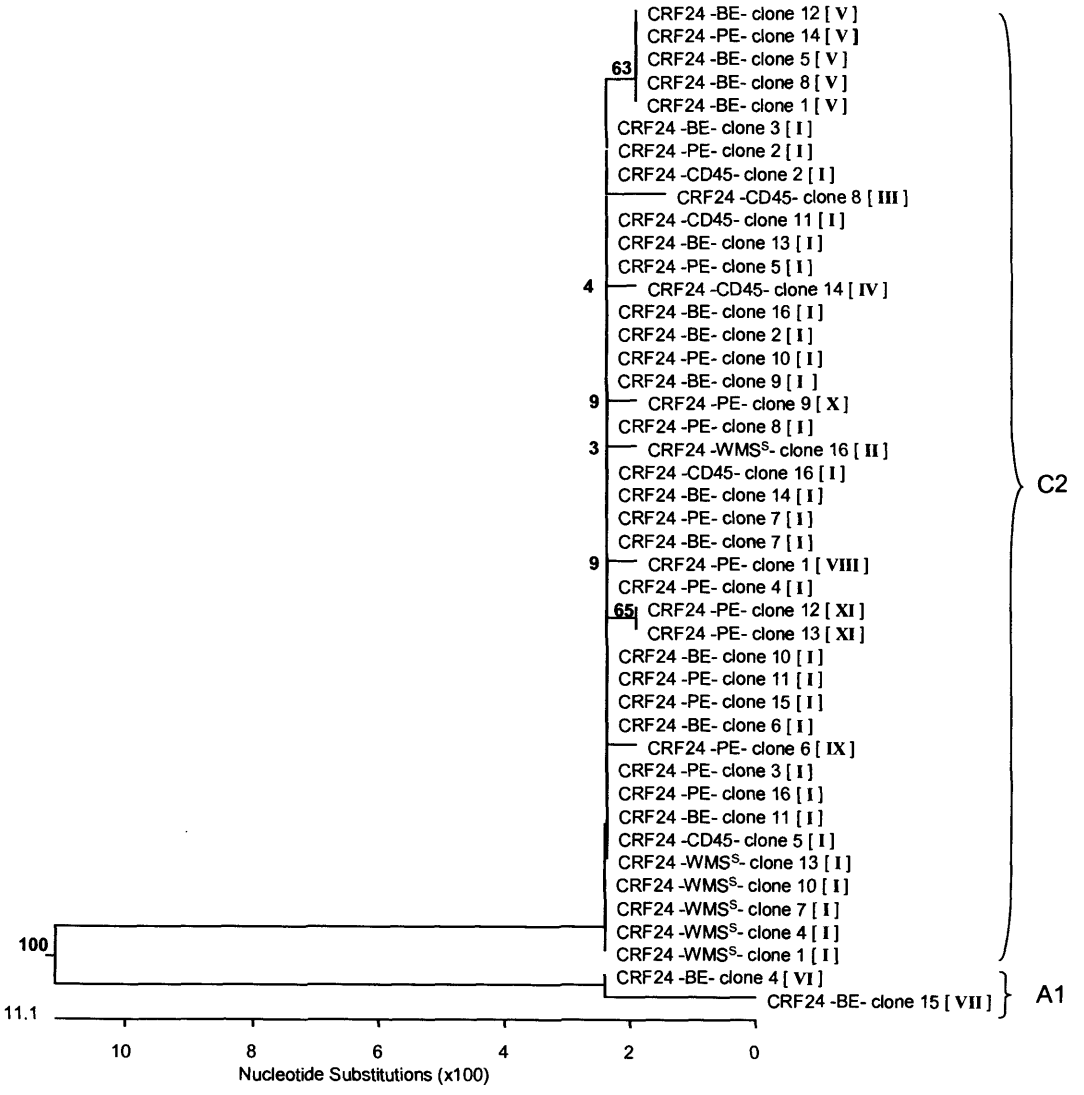
genotype (A1), with a sequence diversity of up to 19.8% from the majority sequence. This A1 sequence was also detected, by direct sequencing, in his CD19+ and CD31+ blood cells (Figure 3.5).

Figure 3.11 (A) Composite of representative DGGE gel photographs accommodating K1/V1 amplicons from 16 clones generated from CD45+ cells, WMS^s, BE and PE amplificates of CRF24; Arabic numerals represent assigned clone numbers, coinciding with lane positions in the gel; colony products with unique sequences are assigned different Roman numerals. (B) Dendrogram showing phylogenetic distribution of K1/V1 consensus derived from (*) indicated clones; Roman numerals in parenthesis denote unique sequences found within specimen; Bootstrapping for 1000 replicates is noted as a percentage at major branch points. (C) A comparison of nucleotide sequences; Roman numerals denote unique sequences and numbers in the parenthesis denote the number of sequenced clones with the unique sequences. Dots indicate residues occupying positions aligned to those of the majority sequence (I) at top.

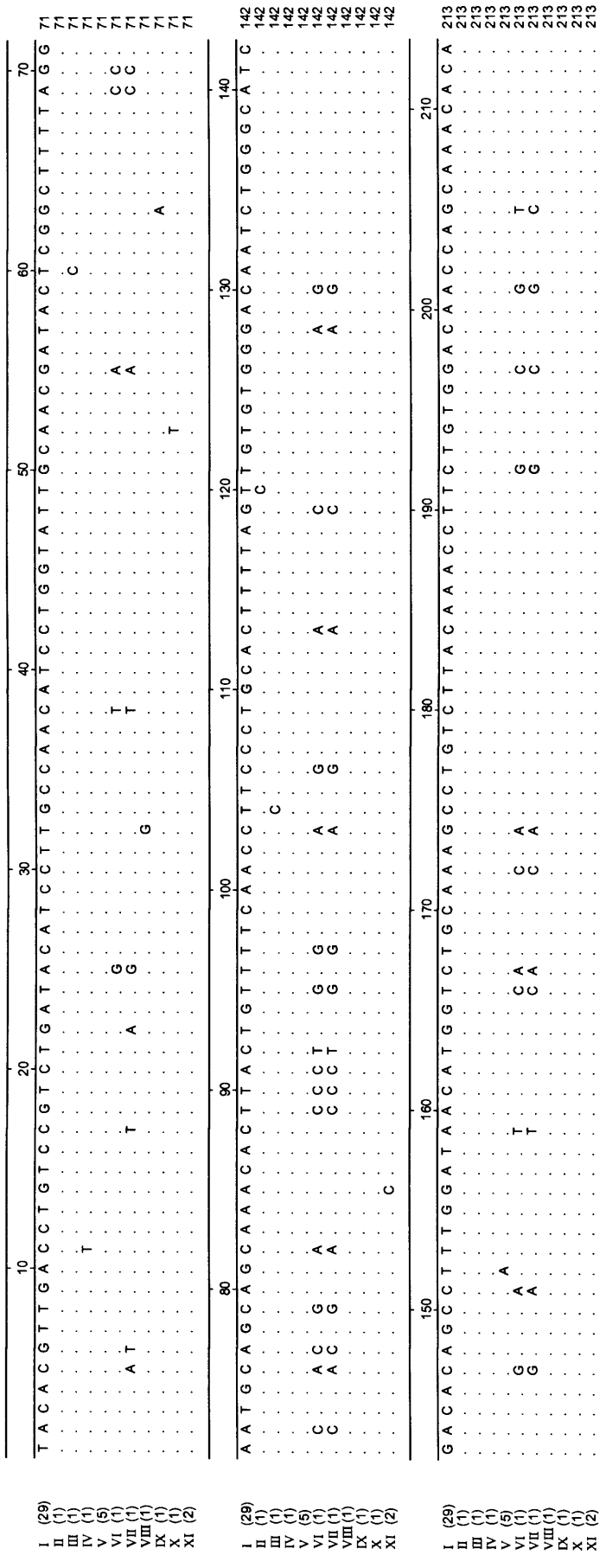
A)



B)


CRF24 -BE- clone 12 [V]
CRF24 -PE- clone 14 [V]
63
CRF24 -BE- clone 5 [V]
CRF24 -BE- clone 8 [V]
CRF24 -BE- clone 1 [V]
CRF24 -BE- clone 3 [I]
CRF24 -PE- clone 2 [I]
CRF24 -CD45- clone 2 [I]
CRF24 -CD45- clone 8 [III]
CRF24 -CD45- clone 11 [I]
CRF24 -BE- clone 13 [I]
CRF24 -PE- clone 5 [I]
4
CRF24 -CD45- clone 14 [IV]
CRF24 -BE- clone 16 [I]
CRF24 -BE- clone 2 [I]
CRF24 -PE- clone 10 [I]
CRF24 -BE- clone 9 [I]
9
CRF24 -PE- clone 9 [X]
CRF24 -PE- clone 8 [I]
3
CRF24 -WMS^S- clone 16 [II]
CRF24 -CD45- clone 16 [I]
CRF24 -BE- clone 14 [I]
CRF24 -PE- clone 7 [I]
CRF24 -BE- clone 7 [I]
9
CRF24 -PE- clone 1 [VIII]
CRF24 -PE- clone 4 [I]
65
CRF24 -PE- clone 12 [XI]
CRF24 -PE- clone 13 [XI]
CRF24 -BE- clone 10 [I]
CRF24 -PE- clone 11 [I]
CRF24 -PE- clone 15 [I]
CRF24 -BE- clone 6 [I]
CRF24 -PE- clone 6 [IX]
CRF24 -PE- clone 3 [I]
CRF24 -PE- clone 16 [I]
CRF24 -BE- clone 11 [I]
CRF24 -CD45- clone 5 [I]
CRF24 -WMS^S- clone 13 [I]
CRF24 -WMS^S- clone 10 [I]
CRF24 -WMS^S- clone 7 [I]
CRF24 -WMS^S- clone 4 [I]
CRF24 -WMS^S- clone 1 [I]
C2
100
CRF24 -BE- clone 4 [VI]
CRF24 -BE- clone 15 [VII]
A1
11.1
10
8
6
4
2
0
Nucleotide Substitutions (x100)

C)



3.3.9.2 CRF 57

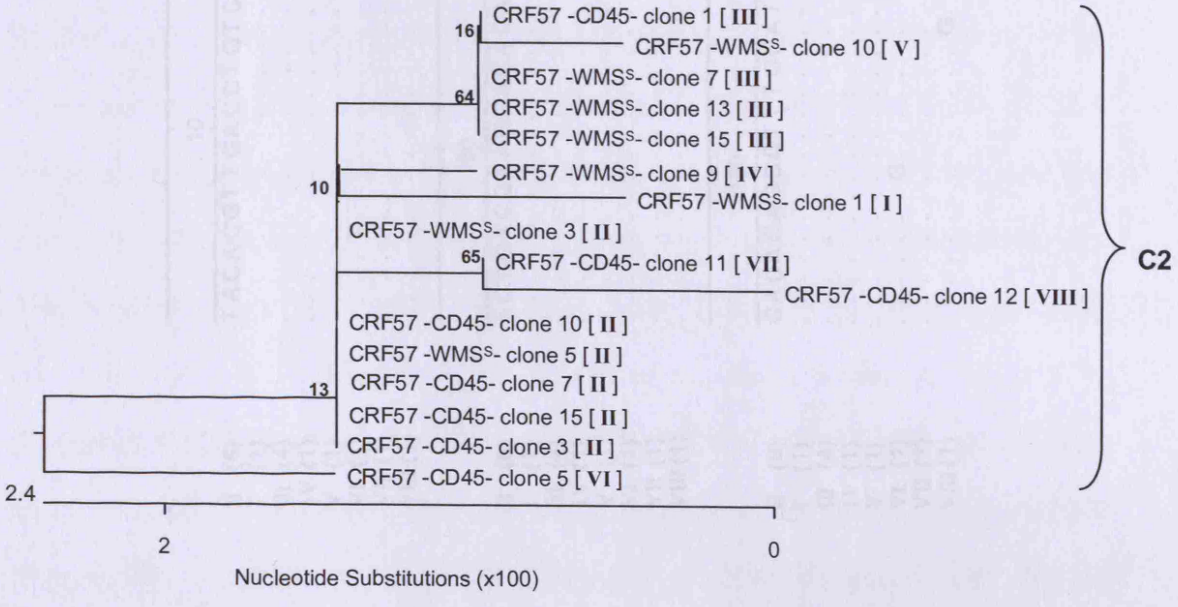
High-fidelity PCR of sample extracts from CD45+ cells and WMS^s of CRF 57 yielded K1/V1 DNA. Clones were generated from which differences in nucleotide sequence could be analyzed by the combined DGGE-sequencing approach. Figure 3.12(A) is a DGGE gel photographic image showing migration distances attained by amplicons generated from clonal inserts; the gel accommodates the two samples with K1/V1 DNA (16 clones/sample). All PCR products that yielded minority bands and some products representing the majority were sequenced (denoted by * in Figure 3.12(A); Arabic numerals correspond to clone numbers). Figure 3.12(B) is a dendrogram depicting the extent of intra- and inter-sample K1/V1 nucleotide sequence diversity. A total of 8 different groups (I to VIII) of sequences were detected. A comparison of the nucleotide sequences is shown in Figure 3.12(C). The consensus ORF K1/V1 sequence from CD45+ cells and WMS^c were identical to the majority sequence group (II), while the consensus ORF K1/V1 sequences from WMS^s and from PE were 0.5% and 15.2% divergent from the sequence group (II), respectively.

Analysis of clones from minority sequences permitted intrasample K1/V1 variability to be further characterized from individual samples. An intra-sample sequence divergence was observed amongst the CD45+ cell ($\leq$3.4%) and WMS^s ($\leq$1.9%) clones. DGGE and nucleotide sequencing analyses detected only one genotype (C2), similar to that detected by direct K1/V1 PCR sequencing of these two samples. Therefore, none of these two samples examined by DGGE carried the minority sequence (A1) previously detected in this patient's PE by direct K1/V1 PCR sequencing.

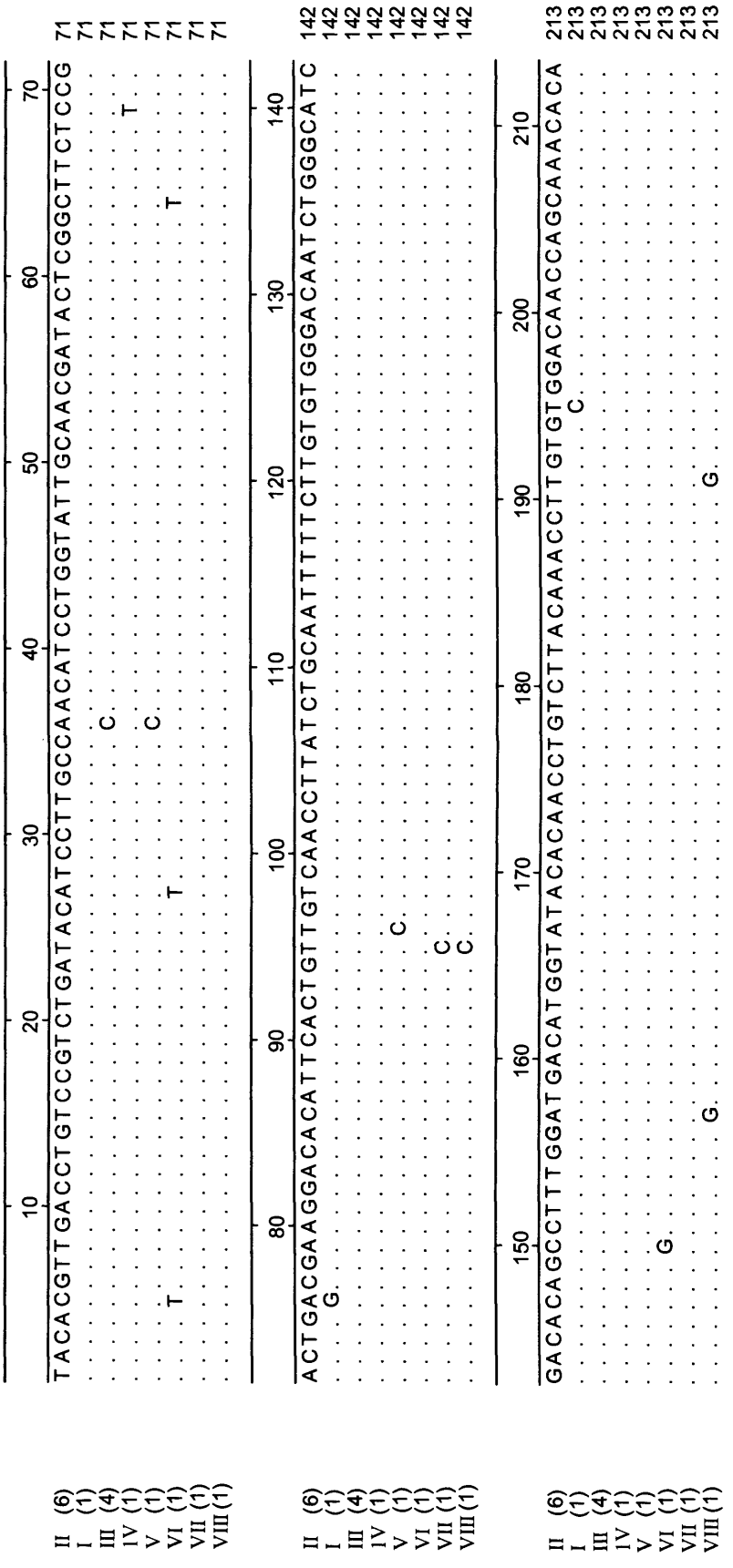
Figure 3.12 (A) DGGE photograph accommodating K1/V1 DNA amplified from 16 clones generated from CD45+ and WMS^S amplificates of CRF57; Arabic numerals represent assigned clone numbers, coinciding with lane positions in the gel; colony products with unique sequences are assigned different Roman numerals, (B) Dendrogram showing phylogenetic distribution of K1/V1 consensus derived from (*) indicated clones; Roman numerals in parenthesis denote unique sequences found within specimen; Bootstrapping for 1000 replicates is noted as a percentage at major branch points, (C) A comparison of nucleotide sequences; Roman numerals denote unique sequences and numbers in the parenthesis denote the number of sequenced clones with the unique sequences. Dots indicate residues occupying positions aligned to those of the majority sequence (II) at top.

A)

B)



C)


10 20 30 40 50 60 70
TACACGTTGACCTGTCCGTCTGATACATCCTTGCCAACATCCTGGTATTGCAACGATACTCGGCTTCTCCG 71
II (6) 71
I (1) . . . . . C. 71
III (4) 71
IV (1) . . . . . T. . . 71
V (1) . . . . . C. 71
VI (1) . . T. . . . . T. 71
VII (1) 71
VIII (1) 71
80 90 100 110 120 130 140
ACTGACGAAGGACACATTCACTGTTGTCAACCTTATCTGCAATTTTTCTTGTGTGGGACAATCTGGGCATC 142
II (6) . . G. 142
I (1) 142
III (4) 142
IV (1) 142
V (1) . . . . . C. 142
VI (1) 142
VII (1) . . . . . C. 142
VIII (1) . . . . . C. 142
150 160 170 180 190 200 210
GACACAGCCTTTGGATGACATGGTATACACAACCTGTCTTACAAACCTTGTGTGGACAACCAGCAAACACA 213
II (6) 213
I (1) . . . . . C. 213
III (4) 213
IV (1) 213
V (1) . . . . . G. 213
VI (1) 213
VII (1) 213
VIII (1) . . . . . G. 213

3.4 Discussion

Epidemiological studies to date have greatly relied on serological data to estimate the prevalence of HHV-8 infection in populations and to study patterns of transmission. In North America and Europe, serological data point to a link between sexual behaviour and risk of HHV-8 infection (Martin *et al.*, 1998; Dukers *et al.*, 2000). However, there is limited seroprevalence and no genoprevalence data relative to HHV-8 in the Saudi Arabian general population and patients with renal disease.

Existing seroprevalence studies from Saudi Arabia have suggested that HHV-8 is not ubiquitous in the Saudi general healthy population: antibodies to HHV-8 were detected in serum samples from 7% of a total of 15 (Qunibi *et al.*, 1998), 3.9% of 258 (Almuneef *et al.*, 2001) and 1.7% of 577 (Alzahrani *et al.*, 2005) people tested. In the present study, IgG antibodies to HHV-8 were detected in the plasma of only 0.6% of the blood donors and none of the pregnant women (0.4% in groups BD and PW combined), proportions that are consistent with the other findings in Saudi Arabia (Table 3.20).

The HHV-8 seroprevalence detected in Saudi Arabian blood donors in this study was also less than that detected in different groups of blood donors from the USA, Southeast Asia, Caribbean (1.3% - 5.2%) and Africa (37.5% - 42%) (Ablashi *et al.*, 1999) tested using the whole virus enzyme-linked immunosorbent assay (ELISA; Advanced Biotechnologies Inc, Columbia, MD, USA) as what is used here, and less than that detected using other assays in healthy controls and blood donors from African countries such as Zambia (ranging between 48% to 71%) (Olsen *et al.*, 1998; He *et al.*, 1998), Congo (up to 82%) (Engels *et al.*, 2000), Botswana (up to 87%) (Engels *et al.*, 2000) and central Africa (22%) (Belec *et al.*, 1998), and from Germany (3.9%) (Lonard *et al.*, 2007), Sweden (20%) (Enbom *et al.*, 2000), Brazil ($\leq$79%) (Biggar *et al.*, 2000; Freitas *et al.*, 2002; Ishak *et al.*, 2007; de Souza *et al.*, 2007b),

Peru (56%) (Mohanna *et al.*, 2007), Argentina (4%) (Perez *et al.*, 2004), Chile (3%) (Perez *et al.*, 2004), Cuba (1.2%) (Kouri *et al.*, 2004), Taiwan (19%) (Huang *et al.*, 2000), northwest China (47%) (Dilnur *et al.*, 2001), USA (up to 26%) (Lennette *et al.*, 1996; Gao *et al.*, 1996b; Baillargeon *et al.*, 2002; Pellett *et al.*, 2003; Martro *et al.*, 2004; Casper *et al.*, 2006) and Mediterranean countries (7.6% to 20%) (Angeloni *et al.*, 1998; Whitby *et al.*, 1998; Calabro *et al.*, 1998; Chatlynne and Ablashi, 1999; Schulz, 2000b; Davidovici *et al.*, 2001; Vitale *et al.*, 2001; Zavitsanou *et al.*, 2007). However, the HHV-8 seroprevalence in Saudi Arabian blood donors, as detected by this study, is more than that detected in one study of blood donors from Japan (0.2%), identified by antibodies against HHV-8 latency-associated nuclear antigen (LANA) using an indirect immunofluorescent assay (Fujii *et al.*, 1999).

When investigating the association in blood donors between HHV-8 seroprevalence and gender, age, blood group, and hepatitis B and hepatitis C infection, no associations were found. Similar findings in relation to hepatitis C infection and gender were observed in other populations (Angeloni *et al.*, 1998; Olsen *et al.*, 1998; Plancoulaine *et al.*, 2000; Iscovich *et al.*, 2000; Hudnall *et al.*, 2003; Perez *et al.*, 2004; Zavitsanou *et al.*, 2007; de Souza *et al.*, 2007b). However, in many populations, especially those in northern Europe, Brazil and North America, the HHV-8 seroprevalence is 2 to 10 times higher in men than women (Whitby *et al.*, 1995; Cesarman *et al.*, 1995b; Lennette *et al.*, 1996; Kedes *et al.*, 1997; Chatlynne and Ablashi, 1999; Greenblatt *et al.*, 2001; Perez *et al.*, 2004). A recent study has however reported a higher HHV-8 seroprevalence amongst Brazilian Amerindian women than men (Ishak *et al.*, 2007).

Studies involving 258 blood donors from Saudi Arabia (Almuneef *et al.*, 2001), 306 blood donors from South Africa (Stein *et al.*, 2004), 339 Amerindians from Brazil (de Souza *et al.*, 2007b), 955 healthy urban employees from Greece (Zavitsanou *et al.*,

2007), 1337 individuals from French Guiana (Plancoulaine *et al.*, 2000), and 100 blood donors from the USA (Hudnall *et al.*, 2003) describe an increase in seroprevalence with age, the latter study also detecting a significant association with ABO blood group B. However, in the current study, despite a sample size of 238, no association was found.

Table 3.20 Summary of Saudi Arabian HHV-8 seroprevalence studies involving healthy volunteers

Study	Nature of controls*	n	Age (yr)			Male (%)	HHV-8 seroprevalence	
			Mean	Range	Std. deviation		(%)	Technique used
Qunibi *et al*, 1998	Healthy volunteers at King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia	15	32.3	NA	10.2	40	7	Immunoblotting assays to detect antibodies to two lytic cycle HHV-8 antigens: p40 and sVCA**
Almuneef *et al*, 2001	Serum submitted to hospital clinical chemistry laboratory and/or blood bank in Riyadh (central region) and Jizan (southern region)	258	47.5	NA	NA	55	3.9	Indirect immuno-fluorescence (lytic HHV-8 antigen) technique and confirmation by immunoblots using acetate induced BC-3 cell extract and the recombinant small viral capsid antigen ORF65
Alzahrani *et al*, 2005	Women attending obstetrics clinics and couples seeking pre-marital genetic counseling at King Fahad Military Medical Complex, Dhahran (Eastern region)	577	27	18 - 44	NA	20	1.7	Immunofluorescence assay testing for antibodies to the latent HHV-8 antigen (LANA)
Present study	Blood donors (accepted and deferred) and pregnant women at the Armed Forces Hospital, Riyadh	238	29.8	17 - 51	7.1	74	0.4	ELISA for detecting anti-body to the majority of HHV-8 structural proteins

* All were Saudis

** p40, an antigen found in infected cells; sVCA, an HHV8-encoded small viral capsid antigen expressed in *Escherichia coli*

In the present study, IgG antibodies to HHV-8 were detected in 16.7% of Saudi Arabian CRF patients, a proportion slightly less than the 29% reported by Qunibi *et al.* (1998), but greater than the 7% reported by Almuneef *et al.* (2001) (Table 3.21). The HHV-8 seroprevalence presently observed is also greater than that in ESRD patients reported from a number of other countries such as Switzerland (6.4%) (Regamey *et al.*, 1998), Greece (7.2%) (Zavitsanou *et al.*, 2006) and Germany (13.7%) (Lonard *et al.*, 2007).

Contrary to the finding of Almuneef *et al.* (2001) that the differences in HHV-8 antibody prevalence between 201 Saudi ESRD patients (7.0%) and 258 individuals without renal disease (3.9%) were not significant, HHV-8 IgG was found in this study to be significantly more frequent in CRF patients than in healthy control individuals. A difference in HHV-8 seroprevalence has similarly been reported recently between ESRD patients prior to transplantation and their corresponding healthy allograft donors, from northwestern Italy (Bergallo *et al.*, 2007), while earlier studies from northern (Luppi *et al.*, 1999), south eastern (Di Stefano *et al.*, 2006), central and south (Cattani *et al.*, 2001) Italy failed to find any significant difference in the HHV-8 seroprevalence between pretransplant patients and controls. The difference of seroprevalence between CRF patients and those without renal disease (BD and PW combined), observed in this study could be attributable to the level and intensity of health care given to CRF patients. Haemodialysis requires regular visits in dialysis units resulting in an increasing rate of nosocomial infections. Uraemic immunodeficiency, exacerbated by the dialysis procedure, may additionally contribute to an increased risk of HHV-8 infection (Haag-Weber and Horl, 1993; Descamps-Latscha and Chatenoud, 1996).

In relation to gender, age, area of origin, duration of dialysis, previous history of organ transplantation, hepatitis B, hepatitis C, therapeutic regimens and oral indices, no

significant associations were found with anti HHV-8 seropositivity in the CRF group. These results are similar to those reported by previous studies (Almuneef *et al.*, 2001; Di Stefano *et al.*, 2006). Other studies found a higher seroprevalence among pre-transplant females (Andreoni *et al.*, 2001) and increasing age (Almuneef *et al.*, 2001; Bergallo *et al.*, 2007).

Table 3.21 Summary of Saudi Arabian HHV-8 seroprevalence studies involving patients with chronic renal failure

Study	Unit location	n	Male (%)	Age (yr)			*Seroprevalence (%)
				Mean	Range	Std. deviation	
Qunibi *et al*, 1998	King Faisal Specialist Hospital and Research Center, Riyadh	14	50	43.9	NA	14.6	29
Almuneef *et al*, 2001	Three centres: 1. King Faisal Specialist Hospital in Riyadh (Central region) 2. King Fahad Hospital in Riyadh (Central region) 3. King Fahad Central Hospital in Gizan (southern region)	201	49	47.1	NA	NA	6.97
Present study	Armed Forces Hospital, Riyadh	72	59.7	57	23 - 83	14	16.7

* The methods used by each research group are the same as those described in Table 3.20

In Saudi Arabian renal allograft recipients, IgG antibodies to HHV-8 were detected in 6.6%, less than that previously reported in Saudi Arabian renal allograft recipients by Qunibi *et al.* (1998) (28%) and later by Alzahrani *et al.* (2005) (18%) (Table 3.22) and in renal allograft recipients from other countries such as USA (50%) (Hudnall *et al.*, 1998), Italy (26%) (Cattani *et al.*, 2001), Iran (25%) (Ahmadpoor *et al.*, 2007) and Germany (22%) (Lonard *et al.*, 2007). However, the detected seroprevalence is greater than that reported from Spain (0.6%) (Garcia-Astudillo and Leyva-Cobian, 2006) and Cuba (0%) (Kouri *et al.*, 2004).

HHV-8 IgG was found to be more frequent in renal transplant recipients than in healthy control individuals. The detection of a higher HHV-8 seroprevalence in Saudi renal allograft recipients when compared to the general population may render them at risk to develop KS and makes it possible that in addition to reactivation of serologically silent opportunistic infections secondary to immunosuppression, specific routes of transmission including infection from the transplanted organ (Luppi *et al.*, 2000a; Kapelushnik *et al.*, 2001; Becuwe *et al.*, 2005) and transfusion of blood or blood products (Blackbourn *et al.*, 1997; Dollard *et al.*, 2005; Hladik *et al.*, 2006; Lefrere *et al.*, 2007) may be potentiated, although transmission via blood transfusion remains controversial and may not apply to non-endemic countries such as Saudi Arabia.

The HHV-8 seropositivity in renal transplant recipients according to age, area of origin, previous history of organ transplantation, location of transplant, nationality of donor, allograft donor status, time that elapsed since the renal transplantion surgery, hepatitis infection, immunosuppressive therapeutic regimens or oral indices, showed no significant differences. These findings support previous studies from Saudi Arabia reporting no significant association between HHV-8 seropositivity in renal transplant patients and the donor's country of origin, the recipient's home region within Saudi

Arabia and the time elapsed since renal transplantation (Almuneef *et al.*, 2001; Alzahrani *et al.*, 2005). The lack of any statistically significant associations between the type or dose of immunosuppressive drugs and the seroprevalence of HHV-8, supports findings from other studies (Alzahrani *et al.*, 2005; Ahmadpoor *et al.*, 2007), but the cross-sectional design of this study had limitations in this regard. Of importance, Anti-HHV-8 IgG was not detected in any RAR patient administered low- or high-dose tacrolimus or in those administered valaciclovir.

The differences between the seroprevalence detected in this study and previous published reports from Saudi Arabia may be explained by the variation in sensitivity and specificity between the different seroprevalence detection techniques used. Whereas the LANA immunofluorescence assay for antibodies against HHV-8 used in the study by Alzahrani *et al.* (2005) has been reported to have lower sensitivity but higher specificity as compared with the immunoblot assay for ORF65 antigen, as used by Qunibi *et al.* (1998). A whole-virus ELISA, as used in this study, has advantages over using individual viral proteins as a source of antigen since the viral lysate contains a broad spectrum of seroreactivity compared to that detected by a single viral protein in ELISA, IFA, or immunoblots.

In conclusion, the results of the present study confirmed high HHV-8 seroprevalence in renal disease patients relative to its prevalence in the general population. This result supports previous data showing the comparatively high prevalence of HHV-8 antibodies in hemodialysis patients (Qunibi *et al.*, 1998; Regamey *et al.*, 1998; Hsu *et al.*, 2002) and renal transplant recipients (Qunibi *et al.*, 1998; Hudnall *et al.*, 1998; Alzahrani *et al.*, 2005) when compared to blood donors from the same country. This association may be confounded by age, as, in the present study, the mean ages of CRF (57 yr) and RAR patients (40 yr) were higher than that of the healthy controls (30 yr). However, in the present study the seroprevalence of HHV-8 in the CRF and

RAR groups was much higher (>40-fold and >16-fold, respectively) relative to the healthy controls. Therefore, it is highly unlikely that such an increase could be attributed to the age factor. Nonetheless, the effect of age cannot be completely excluded. In contrast, the effect of renal disease and induced immunosuppression seem to be more prominent factors.

Table 3.22 Summary of Saudi Arabian HHV-8 seroprevalence studies involving renal allograft recipients

Study	Unit location	n	Male (%)	Age (yr)			Seroprevalence (%)	Immunosuppresives
				mean	range	Std. deviation		
Qunibi *et al*, 1998	King Faisal Specialist Hospital and Research Center, Riyadh	18	56	39.6	NA	15.6	28	prednlsolone plus ciclosporin and azathioprine
Alzahrani *et al*, 2005	King Fahad Military Medical Complex, Dhahran (Eastern region)	150	50.7	41	17 - 72	NA	18	ciclosporin and prednisolone in combination with either azathioprine or mycophenolate mofetil
Present study	Armed Forces Hospital, Riyadh	61	60.7	40	19 - 72	13	6.6	prednisolone in combination with either ciclosporin or tacrolimus. Some patients were additionally administered mycophenolate mofetil

* The methods used by each research group are the same as those described in Table 3.20

Current serologic assays may be insufficiently sensitive to detect low titre antibodies associated with remote HHV-8 infection. This insensitivity raises the possibility that HHV-8 infection is more widespread than can be appreciated from seroepidemiological studies. Thus the necessity for studying genoprevalence arises.

HHV-8 subgenomic DNA was detected in the CD45+ blood cells from nine of the blood donors (9/178; 5.1%) and from none of the pregnant women. The detectability rate in the combined BD and PW group (9/238; 3.8%) was less than that detected by PCR in blood samples of healthy individuals from Japan (12/15; 80%) (Kikuta *et al.*, 1997a), central and southern regions of Italy (3/13; 23%) (Cattani *et al.*, 1998), Central Africa (11/49; 22.5%) (Belec *et al.*, 1998) and Greece (77/800; 9.6%) (Zavos *et al.*, 2005), but more than that reported from France (0/25) (Dupon *et al.*, 1997) and Texas, USA (0/100) (Hudnall *et al.*, 2003). The marked variability in HHV-8 genopositivity between the present study and others may be related to the low copy number of viral DNA present in PBMCs and the differing sensitivity levels achievable in detecting small amounts of HHV-8 DNA (Decker *et al.*, 1996; Blackbourn *et al.*, 1997) or due to the fact that immunocompetent individuals may be able to suppress better the HHV-8 viral load in their blood. It is also possible that detection of HHV-8 DNA in PBMC may be intermittent even in HHV-8 seropositive individuals (Laney *et al.*, 2004). In addition, unlike this study, which included the amplification of both the K1/V1 and KS330 HHV-8 DNA segments, the previous studies cited involved the amplification of ORF26 or ORF72 HHV-8 DNA segments. Moreover, in the present study, blood samples were processed within 24 h after being collected from study participants and were stored at -20°C until DNA extraction was performed. CD45+ cells had been separated from whole blood using a biomagnetic cell separation technique and extracted using a simple commercially available DNA extraction kit (Section 2.3.2). This technique has been previously applied to successfully detect HHV-8 DNA in the CD45+ blood fraction of HIV-1 infected men, blood donors,

patients with KS and their first degree relatives (Leao *et al.*, 2000; Cook *et al.*, 2002a; Cook *et al.*, 2002b; Beyari *et al.*, 2003; Kumar *et al.*, 2007) and is not likely to have resulted in the low levels of K1/V1 DNA detection in the blood samples from the control groups. For these reasons, the low HHV-8 DNA detection rate found in the BD and PW groups very likely reflects very low HHV-8 prevalence in the study groups.

None of the HHV-8 DNA positive blood donors were HHV-8 seropositive and the single donor who was seropositive was not HHV-8 genopositive. The lack of concordance observed between HHV-8 seropositivity and genome detection suggests that low-risk carriers may not mount antibody responses detectable by current serological assays. Hence HHV-8 genome testing may, complementary to serological testing, provide an estimate that more closely mirrors the true prevalence of this virus in low risk populations.

Although haemodialysis patients have been found to be more susceptible to viruses, such as HHV-8 (Hsu *et al.*, 2002), only a few cases of KS have been reported in this group of patients (Herr *et al.*, 2001; Metaxa-Mariatou *et al.*, 2004; Yasar *et al.*, 2007) and studies investigating HHV-8 genoprevalence and the connection between dialysis, HHV-8 DNA, and KS in such patients has not yet been well established (Herr *et al.*, 2001). Therefore, in this study we examined HHV-8 genoprevalence amongst this high-risk group of patients. CRF patients and renal allograft recipients showed positive results for HHV-8 DNA in one or more sample: 22/69 (31.9%) and 11/72 (15.3%) in oral and blood samples of CRF patients, and 12/60 (20.0%) and 15/61 (24.6%) in oral and blood samples of RAR patients. Four CRF (5.8%) and three RAR (5%) patients had detectable HHV-8 in both blood and oral compartments. These genoprevalences of HHV-8 in the blood cell subsets and oral samples of CRF and RAR patients point to a relatively high rate of detection of HHV-8 DNA, which may be related to reactivation due to immune dysfunction.

After adjusting for the unavailable oral samples, HHV-8 DNA was found to be more detectable in the oral samples of CRF patients than in blood, while RARs showed no significant difference in detection rates. A higher detection rate from the oral compartment has been similarly reported in HHV-8-seropositive Egyptian children with acute fever not due to a specific viral exanthema (Andreoni *et al.*, 2002), Kenyan female prostitutes (Taylor *et al.*, 2004) and homosexual men from California (Pauk *et al.*, 2000) with or without KS. In another study, of asymptomatic family members of Malawian patients with KS, HHV-8 genome detection rate was found to be significantly higher in oral than in blood samples: HHV-8 DNA sequences could be detected in mouth-rinses of 18 (27%) out of 67 family members but not in any blood samples (Cook *et al.*, 2002b), probably reflecting the high load of shedding into the oral cavity and the possible lower replicative activity of the virus systemically in the studied groups.

The results based on logistic regression analysis revealed that the detection of HHV-8 DNA in the oral cavity of CRF and RAR patients, in the present study, was not significantly associated with its detection in the blood samples. Similarly, a previous study involving seropositive Zimbabwean women with KS found no significant association between HHV-8 DNA detection in PBMC with its detection in oral samples (Lampinen *et al.*, 2000). Therefore, the oral cavity seems to represent an independent focus of viral residency and perhaps of viral replication.

Reports from different countries have detected HHV-8 DNA in the saliva and the non-lesional oral mucosa of patients with KS or impaired immunity, with or without HIV co-infection. The presence of HHV-8 DNA has been demonstrated in 37% of mouth rinses from 38 Zimbabwean women with KS (Lampinen *et al.*, 2000), 10% of saliva and 40% of oral scrapes from 10 Greek patients with HIV infection and 10 with haematologic malignancies (Triantos *et al.*, 2004), 11.6% of oral epithelial cell

samples from 77 Swiss HIV-infected patients with or without KS (Widmer *et al.*, 2006), 46% of palatal-exfoliate samples, 26% of throat-gargle samples, 21% of mouth-rinse samples from 22 KS patients in Malawi and 67 of their first-degree relatives (Beyari *et al.*, 2003), and in 15% to 75% of saliva and oral swabs from HHV-8 seropositive homosexual American men with and without KS (Koelle *et al.*, 1997; Pauk *et al.*, 2000; Casper *et al.*, 2007). However, the detection rate may be less in immunocompetent individuals, whereas no HHV-8 DNA was detected in whole unstimulated saliva and scrapes from the lingual and buccal mucosa of healthy controls from Greece (Triantos *et al.*, 2004), in oral rinse samples from HIV-negative Zimbabwean women without KS (Lampinen *et al.*, 2000), in saliva from healthy adult volunteers from the USA (Koelle *et al.*, 1997), or in oral tissues of general dental patients from the UK (Di Alberti *et al.*, 1997a). Of note is that this study is the first to examine saliva and oral cells for HHV-8 shedding in ESRD and renal transplant recipients. However, similar samples were not collected from the healthy control group to allow comparison.

The palate has consistently been shown in HIV-coinfected people to be the most common site of development for KS (Flaitz *et al.*, 1997; Gorsky and Epstein, 2000), suggesting that HHV-8 might be particularly tropic for this anatomical site. Moreover, previous studies have detected HHV-8 DNA in the palate in addition to the buccal mucosa of patients with KS (Cook *et al.*, 2002a; Cook *et al.*, 2002b; Beyari *et al.*, 2003), and those with haematologic malignancies or HIV infection (Triantos *et al.*, 2004). The present study supports these findings, but in the context of ESRD patients on haemodialysis and renal allograft recipients. The number of CRF and RAR patients with detectable HHV-8 DNA in their whole saliva (supernate or cellular) samples (16 and 9, respectively) exceeded those with detectable HHV-8 DNA in their buccal (6 and 3, respectively) or palatal (8 and 0, respectively) exfoliates, with samples from whole saliva of CRF patients showing more K1/V1 and KS330

concordantly positive results than other oral samples, implicating a higher copy number of viral DNA. HHV-8 could be detected in both cellular and cell-free salivary fluid, although sometimes disconcordantly, coinciding with previous reports (Blackbourn *et al.*, 1998). Moreover, the limited number of CRF and RAR patients (2 and 0, respectively) with detectable HHV-8 DNA in saliva collected from the parotid duct, supports previous findings indicating that major and minor salivary glands are not important contributors to HHV-8 shedding (Pauk *et al.*, 2000; Corey *et al.*, 2002).

The lower HHV-8 DNA detection rate in BE and PE compared to whole saliva in patients with renal disease, as detected in this study, probably reflects active HHV-8 replication in the oral epithelium other than the buccal mucosa or hard palate or in areas of the palate or buccal mucosa further away from the selected sampling sites. PCR in situ hybridization studies have previously demonstrated a higher concentration of HHV-8-positive cells in the buccal mucosa, with evidence of HHV-8 lytic replication, compared to saliva and scrapings of the lingual epithelial cells from HIV-seronegative and seropositive MSM (Pauk *et al.*, 2000). Moreover, HHV-8 DNA has been detected in a greater number of buccal scrapes than saliva samples from HIV-seropositive and haematologic malignancies patients (Triantos *et al.*, 2004). Additionally, the particularly high WMS^s viral load DNA (119,562,500 genome-copies/ ml) detected in patient CRF 24, whose BE and PE were concordantly positive for both K1/V1 and KS330 HHV-8 DNA, may indicate that HHV-8 in his buccal and palatal cells contributed to the increased WMS^s HHV-8 viral load, supporting the hypotheses that HHV-8 persists and replicates in oral epithelial cells and that these cells are a principal source of the virus in saliva (Vieira *et al.*, 1997; Duus *et al.*, 2004; Johnson *et al.*, 2005).

The rates of detectable HHV-8 DNA in saliva, in the present study (58.3% of seropositive CRF and 66.7% of seropositive RARs), are greater than that reported by

previous studies involving HHV-8 seropositive American MSM (Pauk *et al.*, 2000), Kenyian female prostitutes (Taylor *et al.*, 2004), Brazilian Amerindians (de Souza *et al.*, 2007b) and Ugandan children (Mbulaiteye *et al.*, 2004), which revealed a salivary HHV-8 detection rate of 39%, 32%, 23.7% and 17% respectively. Recently, Casper *et al.* (2007) found 27 of 44 (61%) American HHV-8-seropositive MSM to have detectable HHV-8 in saliva, a proportion higher than that previously reported by others. These differences in HHV-8 DNA detection rates amongst HHV-8 seropositive individuals may be attributed to the difference in the examined study populations, sample size, PCR conditions and target of amplication within the HHV-8 genome.

In the current study, HHV-8 DNA was detected in 35% of both seronegative CRF (20/57) and RAR (20/57) patients, a proportion higher than that detected in the saliva or buffy-coat samples from 87 seronegative Ugandan children (7%) or 102 seronegative mothers (1%) (Mbulaiteye *et al.*, 2004). In other studies, 12% of 24 Malawian KS patients (Beyari *et al.*, 2003) and 34% of 9 Swiss HIV patients (Widmer *et al.*, 2006) with detectable HHV-8 DNA were found to be HHV-8 seronegative. It is most likely that these samples were obtained at the early phase of primary HHV-8 infection (before anti-HHV-8 is mounted to a detectable extent). Follow-up testing of the anti-HHV-8 serostatus would need to be done to monitor for HHV-8 seroconversion.

The results based on the logistic regression analysis revealed that CRF patients with detectable antibodies have 6 times the odds of having HHV-8 in their blood samples and 4 times the odds of having HHV-8 in their oral samples than CRF patients without detectable antibodies. This comparison of geno- and sero-prevalences may allow for assessment of the stage of HHV-8 infection: primary, latent, or lytic, as described earlier. Moreover, in the case of CRF and RAR patients, where a number of patients were both geno- and sero-positive, these individuals may be considered to

be undergoing reactivated or late primary infection. Therefore, testing samples for both geno- and sero-prevalence may be necessary to ensure that HHV-8 infection, regardless of its stage, does not go undetected.

The HHV-8 genopositivity in CRF patients in relation to gender, age, area of origin, duration of dialysis, previous history of organ transplantation, hepatitis B, hepatitis C, therapeutic regimens or oral indices, showed no statistically significant differences between the different groups, although there was a tendency for a higher genoprevalence among female CRF patients than males but this association was not statistically significant.

On the other hand, when investigating possible differences in HHV-8 genoprevalence rates in RAR patients in regard to gender, age, area of origin, previous history of organ transplantation, location of transplant, allograft donor status, time elapsed since the renal transplantation surgery, hepatitis B, hepatitis C, use of immunosuppressive therapeutic regimens, or oral indices, no statistically significant associations were found.

In the present study, there was a tendency for a higher genoprevalence among RAR patients receiving an allograft from a live related donor (61.5%) than among those receiving the allograft from a live unrelated (35%) or cadaver (14.3%) donor; however, this finding was not of statistical significance ($P > 0.05$). The findings of no significant differences in salivary genoprevalence by gender or age are consistent with the results of a recent study reported by Casper *et al.* (2007) who found no association with age, contrary to another study showing a higher salivary HHV-8 DNA detection rate among Amerindian men in Brazil and showing a decrease with age (de Souza *et al.*, 2007b).

The association between malignancy and immunosuppressive therapy is mediated through several mechanisms. Indirectly, immunosuppressive drugs greatly increase the post-transplant risk of malignancy by impairing immunosurveillance and facilitating the action of oncogenic viruses. However, the direct pro- and anti-oncogenic actions of immunosuppressants also play an important role. The cancer-promoting effect of calcineurin inhibitors, independently of depressed immunosurveillance, has been revealed in recent years (Ecder *et al.*, 1998; Guba *et al.*, 2002), and currently, only mammalian target of rapamycin (mTOR) inhibitors have shown simultaneous immunosuppressive and antitumour properties (Guba *et al.*, 2002; Gutierrez-Dalmau and Campistol, 2007).

In this study, two out of three (66.7%) CRF patients receiving low doses of prednisolone were found to be HHV-8 genopositive. This finding may be of importance or merely a coincidence, especially that none of these two patients was seropositive and the single CRF patient receiving a high dose of prednisolone was HHV-8 genonegative. As for the renal allograft recipients, anti-HHV-8 IgG and HHV-8 DNA were respectively detected in 5.9% (3/51) and 43% (22/51) of RAR patients receiving a prednisolone and ciclosporin immunosuppressive regimen compared to 0% and 11.1% (1/9) of those receiving a prednisolone and tacrolimus regimen, suggesting a possibe important role for ciclosporin in HHV-8 infection. However, this association was not statistically significant and the sample sizes were small, therefore further studies comparing groups with a larger number of patients and including other factors, such as the length of exposure to immunosuppressive therapy, may be required to confirm such associations. These lines of study are important as the mechanism by which these different immunosuppressive agents influence the development of KS remains unclear (Cockburn and Krupp, 1989; Hiesse *et al.*, 1995; Rezeig *et al.*, 1997; Farge *et al.*, 1999; Eberhard *et al.*, 1999). Previous studies from Saudi Arabia and Iran failed to find any association between the type and dose of

immunosuppressive drugs and the seroprevalence of HHV-8 (Alzahrani *et al.*, 2005; Ahmadpoor *et al.*, 2007).

Studying the possible link between HHV-8 sero- or geno-prevalence and the patient's area of origin is of importance, as three separate reports from Saudi Arabia indicated that 75% (al Suleiman *et al.*, 1987), 64% (Qunibi *et al.*, 1988) and 32% (Qunibi *et al.*, 1993), of post-transplantation KS cases occurred in patients from the southwestern region of the country, an area in close proximity to Yemen and Africa. However, previous seroprevalence studies from Saudi Arabia did not find an association between HHV-8 seroprevalence and area of residence (Riyadh vs south) amongst a group of Saudi Arabian ESRD patients (Almuneef *et al.*, 2001) and renal allograft recipients (Alzahrani *et al.*, 2005). When comparing the groups of CRF and RAR patients in the current study, with different areas of origin and including only those groups with >1 patient, no statistically significant association between the area of origin and sero- or geno-positivity were found. However, the results do highlight the fact that CRF patients from another area (Hail) located in the north of the country may have a high HHV-8 geno- and sero-prevalence compared to other regions and this finding could be further explored.

At the time of sampling, the majority of RAR patients participating in this study still lived in their area of origin and travelled to Riyadh only to attend hospital follow-up appointments. On the other hand, all participating CRF patients, not originally from Riyadh, had moved to this city, where they have been regularly attending the dialysis unit at the Armed Forces hospital. So, in order to accurately assess the differences in CRF patients HHV-8 sero- or geno-prevalence rates according to the different areas of the country, obtaining samples from CRF patients attending their local dialysis units in their respective area of origin may be more useful.

Although studies of the general population from the USA (Engels *et al.*, 2007) and Greece (Zavitsanou *et al.*, 2007), of children from Uganda (Mayama *et al.*, 1998) and of ESRD patients from Saudi Arabia (Almuneef *et al.*, 2001) found HHV-8 seropositivity to be positively associated with hepatitis B virus infection, a statistically significant association between hepatitis B virus infection and HHV-8 sero- or geno-positivity in either the control or renal disease groups was not found. However, this may be attributed to the low number of participants with hepatitis B in each of the three groups.

To evaluate the sub-cellular distribution of HHV-8 DNA detection and to assure broad representation of the blood subpopulations, a variety of which have previously been reported to support HHV-8 persistence (Table 1.4), immunomagnetic fractioning of CD45+, CD31+ and CD19+ cell subsets was conducted and all fractions tested for HHV-8 infection. A variation in the rate of HHV-8 subgenomic DNA detection in the different blood subsets and in the target of amplication within the HHV-8 genome was observed. A greater number of CRF patients (n=9) had detectable HHV-8 DNA in their CD31+ cells than those with detectable HHV-8 DNA in their CD45+ (n=4) or CD19+ (n=3) cells, which supports previous findings implicating HHV-8 persistence in circulating KS-like spindle cell progenitors (Sirianni *et al.*, 1997a). However, all positive CRF CD31+ cells showed only a single-region positivity, possibly implicating a low copy number of viral DNA in such cells (Decker *et al.*, 1996; Blackbourn *et al.*, 1997). The sub-cellular distribution of HHV-8 DNA differed for the RAR group, where a greater number of RAR patients (n=11) had detectable HHV-8 DNA in their CD19+ cells than those with detectable HHV-8 DNA in their CD45+ (n=3) or CD31+ (n=1) cells. Previous studies have reported HHV-8 DNA detection in CD19+ cells of patients with Castleman's disease (Kikuta *et al.*, 1997b) or with or without KS (Ambroziak *et al.*, 1995; Harrington *et al.*, 1996; Henry *et al.*, 1999; Monini *et al.*,

1999; Pellet *et al.*, 2006). However, none of these studies included patients with renal disease.

CD45 antigen is the most common leucocyte marker and is expressed in all cells of haematopoietic origin except erythrocytes. However, in the present study, a discordant result was observed between the detection of HHV-8 in CD19+ or CD31+ and CD45+ cell subsets in both the CRF and RAR patient groups. This may be owing to the relative paucity of HHV-8 infected B lymphocytes and endothelial cells isolated from whole blood using dynabeads coated with antibodies against CD45+.

In this study, 7/133 (5.3%) of CD45+ cell samples from patients with renal disease yielded HHV-8 subgenomic amplicons compared to 9/238 (3.8%) of CD45+ cell samples from blood donors and pregnant women. This difference was not statistically significant and is contrary to previous findings indicating a higher molecular evidence of HHV-8 activation in the renal transplant population compared with healthy adults (Hudnall *et al.*, 1998). However, the blood genoprevalence comparison was limited to CD45+ cell subsets, as other cell subsets (CD19+ and CD31+ cell subsets) were isolated from whole blood samples of CRF and RAR patients only but not from the BD and PW groups.

None of the 61 renal allograft recipients (including 3 who were both HHV-8 sero- and geno-positive patients) in this study developed KS, although some of the RAR patients had been given immunosuppressive agents for extended periods of time, exceeding 18 years in some cases. Similar findings were detected by Alzahrani *et al.*, 2005, who found none of 150 renal transplant recipients (including 27 HHV-8-seropositive patients) developed KS. That three patients were administered antivirals and nine were administered tacrolimus rather than ciclosporin may have played some role. Moreover, it should be taken into consideration that approximately half of the

RARs in this study were referred from different parts of the country to the Armed Forces Hospital in Riyadh, where renal transplant surgeries were performed, therefore representing many diverse geographic areas and genetic backgrounds.

Previous studies have explored the possible role of genetic and geographic factors. HLA DRB family (mainly HLA DRB1 and HLA DRB5) were found to be important in classic and epidemic forms of KS (Friedman-Kien *et al.*, 1982; Pollack *et al.*, 1983; Gaya *et al.*, 2004; Masala *et al.*, 2005; Dorak *et al.*, 2005), but not in Saudi Arabia (Qunibi *et al.*, 1988). In initial studies, it appeared that KS was associated with the HLA A2 genotype (Qunibi *et al.*, 1988), but this association was not confirmed in additional patients (Qunibi *et al.*, 1993). Another possiibiity is that the increased incidence of KS in Saudi Arabian transplant recipients may be related to variations in locally circulating strains of HHV-8. The ORF K1 region, which exhibits a high degree of sequence diversity, facilitates subtyping. These subtypes correlate with ethnicity and geography (Zong *et al.*, 2002). However, the significance that this diversity brings to the virus is not yet known, but may reflect a positive selection leading to immune selection or evasion (Zong *et al.*, 1999). Moreover, this variation within the genome has not yet been useful clinically. Although strain-specific peptide vaccines are discussed, clinical trials have not been undertaken because the effects of these viruses are often thought to be a secondary consequence of immunosuppression as opposed to primary viral infection (Stebbing *et al.*, 2006).

Direct sequencing studies of the highly variable gene ORF K1 yield data that discriminate between variants better than one that is more conserved, such as KS330. KS330 sequence polymorphism does not provide sufficient resolution for inferences of HHV-8 genotype and strain variation to be made, unless variations are sufficiently wide. Nevertheless, interpreting ORF 26 DNA sequence information may provide considerable value for epidemiological studies and clinical diagnostic

purposes (Zong *et al.*, 2007b). However, because no obvious patterns of linkage were observed in this study between the KS330 and K1/V1 genotypes, direct sequencing of the highly variable region (K1/V1) was chosen when analysing possible association with the various variables.

The majority of HHV-8 K1/V1 amplified from samples obtained from blood donors (C=4, A=2) belonged to C genotype, while the majority of HHV-8 K1/V1 amplified from CRF (A=24, B=1, C=19) and RAR patients (A=22, C=6) belonged to A genotype. These differences were not statistically significant. Subtypes A and C have been known to be the most related (Hayward, 1999; Hughes and Hughes, 2007) with a wide distribution throughout Europe, USA, Asia and the Middle East. In previous studies from Saudi Arabia involving renal transplant patients with associated KS, only A1 and C (C2, C4 and C5) genotypes were detected (Hayward, 1999; Zong *et al.*, 1999). The C2 variant has also been reported to be predominant in classic KS from the USA and Scandinavia (Zong *et al.*, 2002), while the C5 variant has been recently identified in Morrocan classical, post transplant and AIDS-associated KS (Duprez *et al.*, 2006). The current study is the first to report the detection of A2, A5 and B HHV-8 K1 genotypes in the Saudi population. The B ORF K1 subtype has previously been found predominantly in sub-Saharan Africa or in persons of African heritage, while the A5 subtype sequence has been detected in African (Zong *et al.*, 2002; Treurnicht *et al.*, 2002; Hayward and Zong, 2007) and Brazilian samples (Nascimento *et al.*, 2005). The single patient with B genotype ORF K1 sequence was a CRF patient living in a small town outside the capital city Riyadh (appx 500 km) and belonging to an ancient Arabian bedouin tribe; hence, he is unlikely to have any African ancestors. Four patients (CRF23, CRF24, CRF57 and RAR72) had detectable HHV-8 in both their CD45+ cells and WMS, and all of them carried the same genotype in both these two specific samples, regardless of the HHV-8 genotype presented in any of their other genopositive samples. The reason behind this remains unclear.

Although there is constant mixing of cellular components and secreted fluids from various anatomical compartments, the samples obtained in this study may be considered to hold cells and fluids that originate from different compartments. This study determined that some of the compartments sampled can harbour distinct HHV-8 strains, whereas all amplifiable HHV-8 K1/V1 from CD31+ cells (n=9) and PS (n=2) were found to carry A1 genotype. Additionally, A5 HHV-8 K1/V1 genotype was detected only in oral samples (WMS and BE), while detection of A2 genotype was limited to CD45+ cells. However, WMS, BE and CD45+ cells obtained from other patients were found to also carry other genotypes (A1, B, or C2).

For 2 RAR patients, intraperson genotypic differences, arising from nucleotide sequence variations in K1/V1, were found between the oral cavity and blood, while for 3 CRF patients intraperson genotypic differences were found in blood alone. For 1 CRF and 1 RAR patient, intraperson genotypic differences were found in the oral cavity only. These findings support those detecting more than one HHV-8 genotype in the oral cavity and blood (Beyari *et al.*, 2003), reflecting multiple infection. Whether for these 7 people the distinct but closely related variants originated from founder strains that underwent genetic drift after infection or were introduced at different exposure time points cannot be determined due to the cross-sectional nature of this study. Because HHV-8 genomic sequences carried in blood and oral samples from these 7 patients could be either genotypically identical or different, conclusions cannot also be drawn about the selective tropism of HHV-8 strains. Current evidence from other studies suggests that HHV-8 exhibits broad tropism (Dupin *et al.*, 1999; Pauk *et al.*, 2000; Blackbourn *et al.*, 2000).

To detect possible intra-unit HHV-8 transmission in CRF patients, the hypervariable region (K1/V1) was analysed and compared between patients undergoing haemodialysis. Previous studies have documented intra-unit hepatitis C virus

transmission in haemodialysis units using a similar phylogenetic analysis approach, with phylogenetic analysis revealing clustering between patients who were dialyzed during the same shift and in the same area (Hmaied *et al.*, 2007). In the current study, phylogenetic analysis showed a number of CRF patients exhibiting identical K1/V1 sequences. Two pairs of patients were dialyzed during the same shift and in the same room (CRF7/CRF8 and CRF2/CRF15), while others were dialyzed during the same shift but in different rooms (CRF46/CRF48 and CRF7&8/CRF61). These findings suggest possible nosocomial spread of HHV-8 among patients undergoing haemodialysis. Spread could have been facilitated by sharing articles between patients and/ or via nurses and medical staff, especially that these patients were accommodated at close quarters housing patients who are infectious for the virus with those who are unprotected from primary infection, re-infection or super-infection. That this mode of transmission occurred is further supported by the finding that CRF patients were found to have high HHV-8 seropositivity and oral genopositivity rates.

The finding that the three RAR patients carrying more than one HHV-8 K1/V1 genotype had undergone only one renal transplant surgery and received the kidney allograft within the past 2 yr was unexpected. The patients may have been exposed to simultaneous pre- or post- transplant primary infection with different HHV-8 strains or were infected with only one strain prior to transplant after which they became exposed post-transplant to HHV-8 superinfection with a different strain due to immunosuppression or via the transplanted organ. However, as this was a cross-sectional study and no pre-transplantation samples were available for analysis, no definitive conclusions can be reached. Surprisingly, two of the three RAR patients carrying more than one genotype received the allograft from a relative and assuming that the superinfection was transmitted via the transplanted organ, it could be considered that relatives were more likely to carry the same HHV-8 genotype, especially if they had contracted infection from one another.

Immunocompetent individuals infected with multiple viral strains have been identified for CMV and EBV (Meyer-Konig *et al.*, 1998; Srivastava *et al.*, 2000; Walling *et al.*, 2003). Multiple infection with HIV-1 and HIV-2 is also possible (Steain *et al.*, 2004). Although it is unclear how immunological responses generated by the host after first exposure protect against HHV-8 infection from subsequent exposures, it would appear that humoral responses may not be neutralizing, because HHV-8 can actively replicate in people who are seropositive for the virus (Tedeschi *et al.*, 2001). Accordingly, people growing up and living in regions where HHV-8 is hyperendemic are potential hosts to multiple HHV-8 strains. However, few studies have reported multiple infection of HHV-8 (Gao *et al.*, 1999; Beyari *et al.*, 2003).

The present study focuses on whether intrahost HHV-8 subgenomic sequence polymorphism exists among and within oral and blood samples of Saudi patients with renal disease and the extent of such polymorphism. Because direct PCR sequencing will not identify minor populations of the virus, it was necessary to study a large number of cloned PCR products to investigate the level of intra-individual diversity present. The DGGE approach was selected. DGGE was developed primarily for the detection of genetic mutations in human disease and can reliably detect single base pair mutations. The technique is based on variations in the electrophoretic mobility of double-stranded DNA through a gradient of increasing concentrations of formamide or urea denaturants (Myers *et al.*, 1987). The DNA fragment will migrate through the gradient gel until it reaches a point where the concentration of denaturants is equal to the melting temperature, T_m, of its lowest melting domain. This will cause the fragment to unravel, thus retarding its progress through the gel. The T_m of any domain is dependent on its nucleotide composition and mutations in low melting point domains and as few as one nucleotide will affect the fragment's mobility and ultimate position in the gel. However, distinct electrophoretic patterns are not only determined by the number of different nucleotides between molecules, but they are also affected

by the type of base involved and its position in the fragment (Harris and Teo, 2001). Use of a 'GC Clamp', a high GC rich domain introduced during PCR to one end of the fragment, increases sensitivity by preventing the DNA from becoming completely denatured under DGGE conditions (Sheffield *et al.*, 1989; Fodde and Losekoot, 1994). This process will occur on a different position in the gel, when one or more base pairs are substituted or deleted.

In this study, DGGE was used to rapidly screen a large number of cloned PCR products derived from the K1/V1 region of HHV-8 in selected study participants. This region was chosen because of its hypervariable nature and because ORF K1/V1 sequence data was already available for each individual. Using this technique, up to 40 clones could be analysed from one gel. Subsequent sequencing of selected clones was necessary to determine both the sequence of the majority of clones isolated from a single individual and to investigate the level of intra-individual diversity present.

During sequencing, *Taq* polymerase error can be minimised by either sequencing directly from a PCR product to obtain the consensus sequence of all amplicons or by taking the majority sequence from a number of cloned PCR products. In studies where viral diversity is to be investigated by examining the sequence of individual cloned amplicons, the effect of *Taq* polymerase error can be magnified and maybe falsely interpreted as true viral diversity, especially that sporadic mutations often occur at a rate equivalent to *Taq* polymerase error (Smith *et al.*, 1997a). Therefore, it was necessary to evaluate the impact that *Taq* polymerase error may have on the apparent presence of variant HHV-8 genomes in an individual compared to EXPAND.

Clones amplified from the BCBL-1 cell lines were analysed using both the EXPAND system, designed to introduce fewer polymerase induced errors, and *Taq* polymerase

by DGGE. The results from this experiment suggested that when using the EXPAND system, only 1 of 11 (9%) BCBL-1 clones had a sequence differing from the majority, while *Taq* polymerase produced a significantly greater number of variant clones (6 of 11; 54.5%), potentially overestimating the extent of viral diversity. Moreover, 5 out of 11 (45.5%) clones generated using *Taq* polymerase to amplify ORF K1/V1 PCR products followed by the use of EXPAND during colony PCR had a sequence differing from the majority. All of these clones were verified by sequence analysis and were found to differ by 1 to 3 bp from the majority.

The use of high-fidelity polymerases in this study was crucial to enquiring if multiple HHV-8 infection had occurred in patients with renal disease. Intraperson genotype and subgenotype K1/V1 sequence differences were found among samples of 7 patients. Because direct PCR sequencing may not identify minor populations of the virus, it was necessary to study a large number of cloned PCR products to investigate the level of intrasample diversity present. Using DGGE, multiple ORF K1/V1 clones from two CRF patients, from whom non-identical ORF K1/V1 sequences were recovered and K1/V1 amplification using EXPAND was possible, were screened simultaneously. The high-fidelity (EXPAND) PCR was applied to the sample extracts. However, high-fidelity PCR was relatively inefficient in generating amplicons from other CRF patients of interest, explaining why, of extracts from CRF patients from whom intersample K1/V1 sequence differences could be evaluated by direct sequencing, K1/V1 amplicons could be generated from sample extracts from only 2 people.

DGGE and nucleotide sequencing of clones permitted intrasample K1/V1 variability to be further characterized from individual samples. Analyses revealed genotypic differences and confirmed findings from direct K1/V1 PCR sequencing that CRF 24 carried >1 genotype. For the BE sample, direct sequencing of K1/V1 generated

sequences assignable as genotype C2, but clonal analysis revealed carriage of minority sequences belonging to A1 genotype. This A1 sequence was also detected, by direct sequencing, in his CD19+ and CD31+ blood cells. Why this minority sequence was detected in the buccal sample but not the palatal, CD45+ cells or WMS^s is unclear. On the other hand, DGGE and nucleotide sequencing analyses of CRF 57 CD45+ cells and WMS^s clones detected only one genotype (C2), similar to that detected by direct K1/V1 PCR sequencing of these two samples.

The findings from this study are supported by other reports (Gao *et al.*, 1999; Beyari *et al.*, 2003) but contrast with some studies in individuals with (Meng *et al.*, 2001; Stebbing *et al.*, 2001; Zong *et al.*, 2002) and without (Mbulaiteye *et al.*, 2006) KS, which have not revealed evidence for intra-person variation in HHV-8 sub-genomic sequences. The paucity in the number of reports of mixed HHV-8 infection may be due to a combination of factors. First, homosexual men, involved in most KS studies, are exposed to HHV-8 later in life, rather than early in childhood, and favour HHV-8 strains transmitted via sexual routes, rather than those transmitted nonsexually within the household, renal units or community. Second, in previous studies examining for HHV-8 genomic polymorphisms, the PCR products were directly amplified from the samples (Poole *et al.*, 1999; Gao *et al.*, 1999; Codish *et al.*, 2000; Meng *et al.*, 2001; Stebbing *et al.*, 2001; Zong *et al.*, 2002); in doing so, the existence of minor virus populations would have been revealed only if they constituted a relatively large fraction (>20%) of the entire population (Ngui and Teo, 1997). When consensus sequencing protocols are applied, the sequence information obtained will not differentiate minority sequences from the sequence of the dominant variant. Third, previous studies have been largely confined to patients with KS or patients who are at risk of developing KS. When sequence polymorphism of subgenomic HHV-8 DNA in the blood is sought from such people, the tendency is for none to be found, particularly when consensus sequencing is applied, because the high HHV-8 genome

load (Tedeschi *et al.*, 2001) favours the generation of sequences reflecting the dominant variants.

The design of the current study, owing to its cross-sectional nature, precludes resolution of whether concurrent HHV-8 infections in the patients resulted from co-infection or super-infection and was confined to examination of HHV-8 carried in the blood and the mouth. Sampling of other body sites may reveal more evidence of multiple HHV-8 carriage in patients with renal disease.

Chapter 4

Salivary HHV-8 Shedding in KS-experienced Renal Allograft Recipients

4.1 Introduction

The previous chapter describes an investigation into the extent of serologic as well as genomic prevalences of HHV-8 infection in the Saudi Arabian general population, patients with end-stage renal disease, and renal allograft recipients without KS. Shedding of HHV-8 via different anatomical compartments, and polymorphisms in HHV-8 subgenomic sequences were characterised. It was revealed that a higher HHV-8 seroprevalence existed in Saudi Arabian patients with renal disease without a history of KS, when compared to the general population. On the other hand, no statistically significant difference was found with regard to HHV-8 DNA prevalence rates in CD45+ cell samples. While HHV-8 DNA was significantly more detectable in the oral samples of CRF patients than in their blood, a similar difference could not be detected in renal allograft recipients. With regard to HHV-8 polymorphisms in patients with renal disease, evidence was provided of inter- and intra-compartmental multiple HHV-8 infection.

KS is the most common malignancy in renal allograft recipients in Saudi Arabia, comprising 87.5% of 16 neoplasms in one report (Qunibi *et al.*, 1988) and 70% of 37 neoplasms in another (Qunibi *et al.*, 1993), both studies having been conducted in the King Faisal Specialist Hospital and Research Centre in Riyadh. The single report from the Riyadh Armed Forces Hospital found KS constituting 76% of 46 post-transplant neoplasms (al Sulaiman and al Khader, 1994). KS was found to affect 3.4% of 350 (al Suleiman *et al.*, 1987), 5.3% of 263 (Qunibi *et al.*, 1988) and 4.7% of 730 (al Sulaiman and al Khader, 1994) Saudi Arabian renal allograft recipients.

This chapter reports on studies into the extent of blood and oral HHV-8 shedding in a group of Saudi Arabian KS-experienced renal allograft recipients. The presence of multiple HHV-8 infection was also determined by comparing the genomic diversity of HHV-8 found in the mouth with that in other body compartments.

4.2 Patients, materials, and methods

4.2.1 Study population

The study group was 5 male Saudi Arabian renal allograft recipients with histologically confirmed KS (the RAR-KS group) seen at the Armed Forces Hospital in Riyadh, Saudi Arabia. The patients came from five different areas of the Kingdom of Saudi Arabia (Albaha, Aseer, Jizan, Qaseem and Hail). The recipients were diagnosed with KS between 1999 and 2004. All had received live renal allografts between 1982 and 2001. The kidney donors were of 4 different nationalities (Table 4.1). The mean age of recipients was 56 yr and mean time between transplant and diagnosis of KS was 9.7 yr. KS affected the skin of the lower limbs in 4 renal allograft recipients and the eyelid in 1 patient. For 1 patient (patient 5), KS lesions were clinically evident at the time of study, while for the other patients the clinically evident lesions had cleared. All patients had been on a dual immunosuppressive regimen (ciclosporin and prednisolone) at the time of KS diagnosis. Two patients (patients 1 and 4) were treated by surgical removal of the KS. In another 2 patients (patients 2 and 3), KS regression followed the discontinuation of ciclosporin. The fifth patient (patient 5) underwent chemotherapy (a combination of dexamethasone, doxorubicin, bleomycin, vinblastine and dacarbazine) for KS in addition to having the ciclosporin dosage reduced; he was still undergoing treatment at the time of sample collection. Renal graft function was adequate in 3 patients while the remaining 2 were returned to dialysis (Table 4.1).

Table 4.1 Demographic and clinical characteristics of study patients

Patient	Age* (yr)	Nationality of donor	Date of transplant	Interval between transplant and KS diagnosis (months)	Site of skin lesion(s)	Therapy for KS	Outcome
1	43	Indian	1993	84	Right upper eye lid	Surgically removed	Graft loss; on haemodialysis
2	65	Indian	1992	89	Right lower limb	Discontinuation of ciclosporin	Graft loss; on peritoneal dialysis
3	54	Saudi	1982	226	Right lower limb	Discontinuation of ciclosporin	Graft function maintained
4	59	Pakistani	2001	6	Right lower limb	Surgically removed	Graft function maintained
5	57	Jordanian	1990	174	Right lower limb	Reduction of ciclosporin + chemotherapy	Treatment still in progress

* At time of sample collection

4.2.2 Sample collection

After a standardized interview and oral examination, as detailed in Table 2.1 and Appendix 1, matched oral, blood and biopsy samples were obtained as described in Sections 2.2.1, 2.2.2, 2.2.3 and 2.2.4. The oral samples were collected immediately after the blood samples were obtained. The mean time between start of KS treatment and sample collection for the 5 patients was 3.5 yr (range: 0.5 – 5.2 yr).

4.2.3 Sample processing

After separation of plasma from the blood, the CD45+, CD31+ and CD19+ cell subsets were immunomagnetically fractionated (Kumar *et al.*, 2007) as described in Sections 2.2.1.1 & 2.2.1.2. The cellular and supernate fractions of WMS (WMS^c and WMS^s, respectively) were separated by low-speed centrifugation. BE and PE were similarly pelleted. Following aspiration of the supernate, WMF^c, BE and PE were resuspended in 1 ml of PBS, and stored at -20°C until required. DNA was extracted from plasma, blood cell subsets and oral samples as described in Section 2.3.2. Paraffin-embedded tissue from KS biopsy samples was sectioned (Section 2.2.4) and DNA extracted (Section 2.3.2). The presence of DNA in each extract was verified by amplifying a fragment of the β-globin gene as described in Section 2.3.3.

4.2.4 Sequencing analysis of DNA amplified from KS330 and K1

The 211-bp KS330 was amplified from sample extracts by use of nested PCR (Section 2.3.4). The 247-bp K1/V1 was amplified from sample extracts by nested PCR (Section 2.3.5.1). In addition, all HHV-8 K1/V1 or KS330 DNA-positive samples were amplified for long K1 by nested PCR (Section 2.3.5.2). Extracts were tested at least twice for KS330, K1/V1 and long K1 DNA, and considered positive when PCR repeatedly yielded products. PCR products were sequenced, raw DNA sequence data were analyzed and phylogenetic analyses were performed as detailed in Sections 2.3.10 & 2.3.11.

4.2.5 Intra-person K1/V1 sequence differences as revealed by DGGE

Saliva and biopsy samples were subjected again to nested PCR by use of the EXPAND High Fidelity PCR System as described in Section 2.6.1. The PCR products were then purified directly from the second-round PCR product (Section 2.3.8). Clones were generated from both saliva and biopsy K1/V1 purified DNA (Section 2.3.9). From 15 colonies from each amplificate, another round of PCR was done using a clamping primer as described in Section 2.6.2. The PCR products were subjected to DGGE (Beyari *et al.*, 2003) as detailed in Sections 2.6.3, 2.6.4 and 2.6.5, followed by nucleotide sequencing (Section 2.3.10). Raw DNA sequence data were analyzed and phylogenetic analyses were performed as detailed in Section 2.3.11.

4.2.6 Screening for and characterising inter- and intra-sample long K1 sequence differences

Long K1 PCR products from all saliva and biopsy samples were isolated and purified from agarose gels as described in Section 2.3.8. Purified DNA fragments were visually verified by gel examination before cloning. The long K1 gene fragment was cloned into vectors as described in Section 2.3.9. Colonies were screened by PCR using long K1 second-round primers. At least 10 clones from each amplificate were sequenced after plasmid DNA purification. Clones were sequenced using forward and reverse M13 primers and the inner primers (K1b-f & K1b-r). Sequencing was performed using the Beckman-Coulter CEQ2000 automated capillary array sequencer (Section 2.3.10). Raw DNA sequence data were analyzed using Seqman software (DNAstar) (Section 2.3.11). For the purpose of this study, subgenotypic differences were considered to be significant if the divergence between long K1 sequences was $\geq 5\%$. This accommodates base incorporation errors due to *Taq* polymerase error-prone activity and suggests that the differences are due to sequences that originated from distinct virus strains.

PCR products were also subjected to DGGE, with the GC-clamp K1/V1 PCR reaction (using EXPAND) incorporating purified DNA generated from cloning long K1 amplicons into vectors. This allowed the resulting K1/V1 PCR amplicons to be representative of the VR1 of the long K1 colonies.

4.2.7 Quantitative PCR

WMS^{s} extracts from 3 patients, showing concordantly positive results for K1/V1 and ORF 26 DNA, were referred to the National Centre for Infectious Diseases, Centres for Disease Control and Prevention, Atlanta, GA, where a quantitative, fluorescence-based, real-time PCR was applied (Section 2.4).

4.2.8 Anti HHV-8 IgG detection

The Advanced Biotechnologies ELISA was applied to plasma samples (Section 2.5).

4.3 Results

4.3.1 HHV-8 DNA detection and intra-person sequence diversity

4.3.1.1 Detection rates

Patient 2 was the person with the most samples positive for HHV-8 DNA, and patient 5 was the one without HHV-8 DNA detected in any oral samples (Table 4.2; Figure 4.1). KS330 DNA could be amplified from all tested biopsy samples and a varying number of oral samples. However, none of the patients showed positive results for KS330 DNA in their tested blood samples. For KS330 DNA in oral samples, the detection rates were as follows: 60% (3/5) for WMS^s, 40% (2/5) for WMS^c, 20% (1/5) for BE and PE, 0% (0/5) for PS.

K1/V1 DNA could be amplified from all plasma and biopsy samples, variably from the oral samples, and not from the blood cell subsets. For K1/V1 DNA in oral samples, the detection rates were as follows: 80% (4/5) for WMS^s, 40% (2/5) for WMS^c, and 20% (1/5) for PS, BE and PE. Seven oral samples (29%) were concordantly positive for KS330 and K1/V1 DNA (Table 4.2; Figure 4.1).

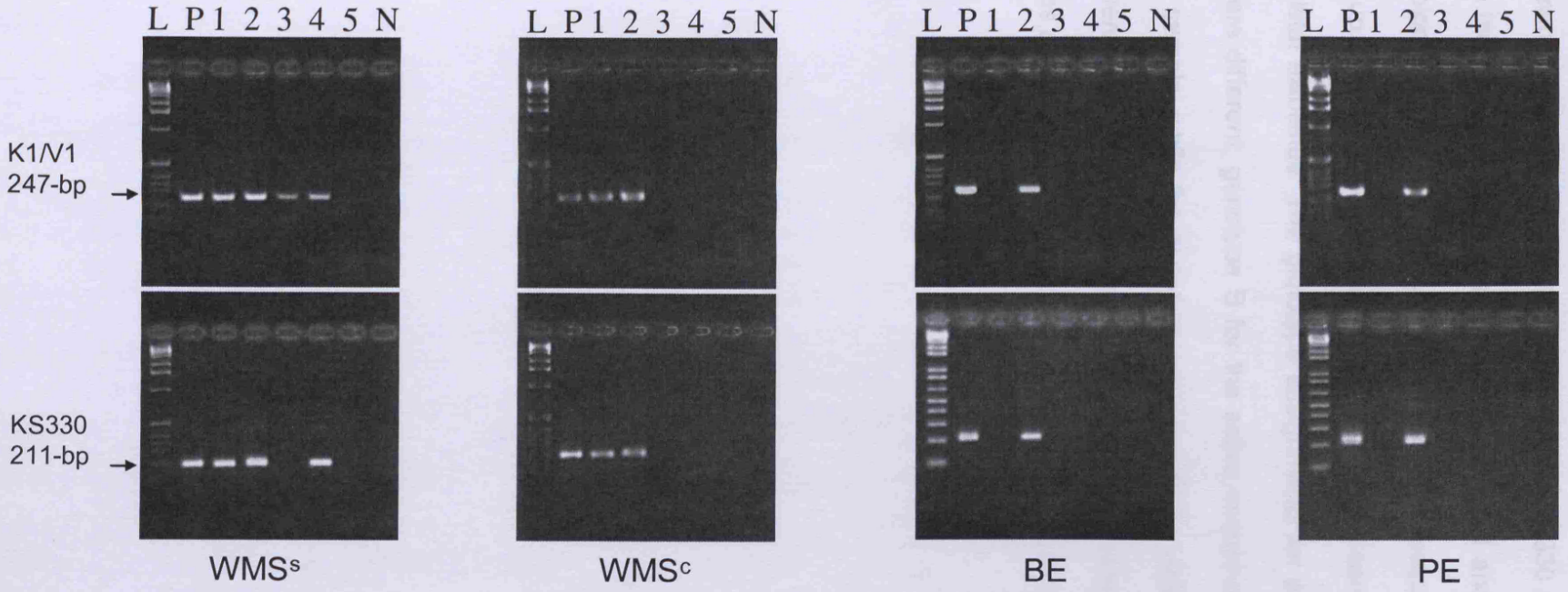
All biopsy and majority of oral samples, positive for K1/V1, were concordantly positive for long K1 DNA. However, none of the patients showed positive results for long K1 DNA in their blood samples.

Table 4.2 Summary of HHV-8 DNA and anti-HHV-8 IgG results, viral loads and oral indices

Patient	HHV-8 DNA																					WMS^s Genome load (copies/ml)	Anti- HHV-8	Plaque index	Gingival index	Bleeding Index
	Blood					Oral													KS Biopsy							
	Cells*		Plasma			WMS^s			WMS^c			PS			BE			PE								
	KS330	K1/V1	KS330	K1/V1	long K1	KS330	K1/V1	long K1	KS330	K1/V1	long K1	KS330	K1/V1	long K1	KS330	K1/V1	long K1	KS330	K1/V1	long K1	KS330	K1/V1	long K1					
1	-	-	-	+	-	+	+	+	+	+	-	-	-	nt	-	-	nt	-	-	nt	+	+	+	1238	+	2.08	1.21	0.04
2	-	-	-	+	-	+	+	+	+	+	+	-	-	nt	+	+	-	+	+	+	+	+	+	2198250	+	0.92	1.25	0.25
3	-	-	-	+	-	-	+	+	-	-	nt	-	+	-	-	-	nt	-	-	nt	+	+	+	nt	+	1	1.46	0.29
4	-	-	-	+	-	+	+	+	-	-	nt	na	na	na	-	-	nt	-	-	nt	+	+	+	3778	+	1	1	0
5	-	-	-	+	-	-	-	nt	-	-	nt	-	-	nt	-	-	nt	-	-	nt	+	+	+	nt	+	0.83	1	0

*CD45+, CD31+ and CD19+ subsets. +, HHV-8 DNA amplified; -, HHV-8 DNA not amplified; nt, not tested; na, sample not available; WMS^s, whole-mouth saliva supernate fraction; WMS^c, whole-mouth saliva cellular fraction; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate

Figure 4.1 Representative agarose gel electrophoresis images of the PCR amplification products

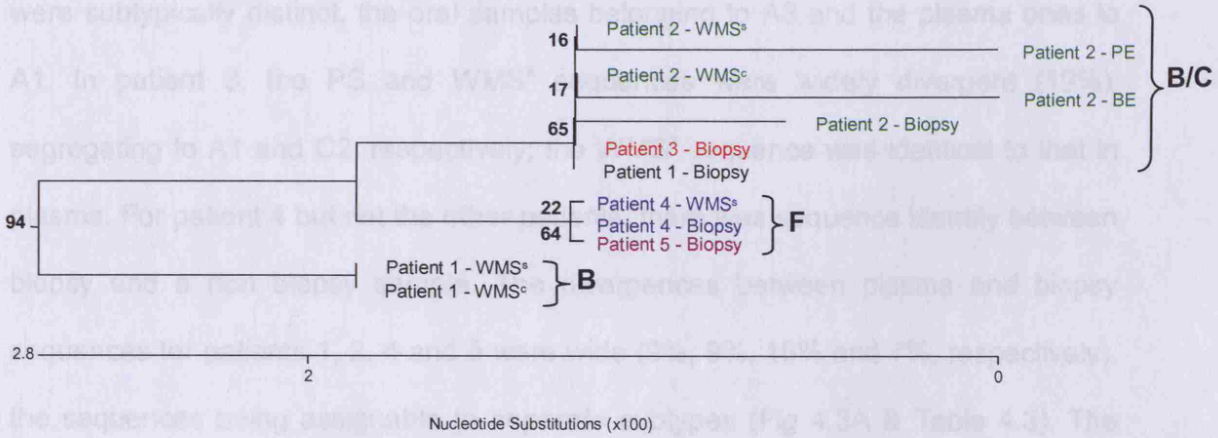


Abbreviations: L= DNA marker band (1Kb); P= positive control; N= negative control; 1, 2, 3, 4 & 5= patient number; WMS^S, whole-mouth saliva supernate fraction; WMS^C, whole-mouth saliva cellular fraction; BE, buccal exfoliate; PE, palatal exfoliate

4.3.1.2 KS330 nucleotide sequence diversity

A dendrogram displaying the diversity of consensus KS330 sequences, and their genotypic assignments is shown in Figure 4.2. Identical KS330 sequences were observed between the WMS^s and WMS^c samples for patients 1 and 2, and between the WMS^s and biopsy sample of patient 4. Intra-person sequence diversities, however, reached up to 2.5% between patient 1 biopsy and oral samples, and patient 2's buccal and palatal exfoliates. The genotype assignments for patient 1 oral and biopsy samples were different; genotype B for the saliva sequences and genotype B/C for the biopsy sample, while the genotype assignments for patient 2 buccal and palatal exfoliates were similar (B/C) (Figure 4.2; Table 4.3). The extent of intra-person sequence diversities relative to the KS biopsy sequence of all patients is summarised in Table 4.4.

Figure 4.2 Predicted phylogenetic distribution of RAR-KS consensus KS330 sequences. Bootstrapping for 1000 replicates is noted as a percentage at major branch points.



4.3.1.3 K1/V1 nucleotide sequence diversity

A dendrogram displaying the diversity of consensus K1/V1 sequences, and their genotypic assignments is shown in Fig 4.3A. Between the WMS^s and WMS^c samples of patient 1, and among the oral samples of patient 2, intra-person sequence diversities were narrow (≤0.5%). The oral and plasma samples of these 2 patients were subtypically distinct, the oral samples belonging to A3 and the plasma ones to A1. In patient 3, the PS and WMS^s sequences were widely divergent (19%), segregating to A1 and C2, respectively; the WMS^s sequence was identical to that in plasma. For patient 4 but not the other patients, there was sequence identity between biopsy and a non biopsy sample. The divergences between plasma and biopsy sequences for patients 1, 2, 4 and 5 were wide (9%, 9%, 16% and 7%, respectively), the sequences being assignable to separate subtypes (Fig 4.3A & Table 4.3). The extent of intra-person sequence diversities relative to the KS biopsy sequence of all patients is summarised in Table 4.4. K1/V1 sequences have been deposited in EMBL Nucleotide Sequence Database (accession numbers AM422987 to AM423005).

4.3.1.4 Long K1 nucleotide sequence diversity

A dendrogram displaying the diversity of consensus long K1 sequences, and their genotypic assignments is shown in Fig 4.3B. An alignment of the long K1 amino acid sequences is shown in Fig 4.4. For patient 2, the divergences among his oral and lesional KS sequences were narrow (<0.3%), and both could be assignable to genotype C5, contrasting with A3 assigned following K1/V1 sequencing. A 15-bp deletion was observed in the C5 sequences (Fig 4.4). For patient 4, the WMS^s and biopsy sequences were identical. Table 4.4 summarises the extent of intra-person sequence diversities relative to the KS biopsy sequence of each patient. Long K1 sequences have been deposited in EMBL Nucleotide Sequence Database (accession numbers AM423127 to AM423139).

Figure 4.3 Diversity of RAR-KS consensus K1/V1 sequences (A) and consensus long K1 sequences (B). Bootstrapping for 1000 replicates is noted as a percentage at major branch points.

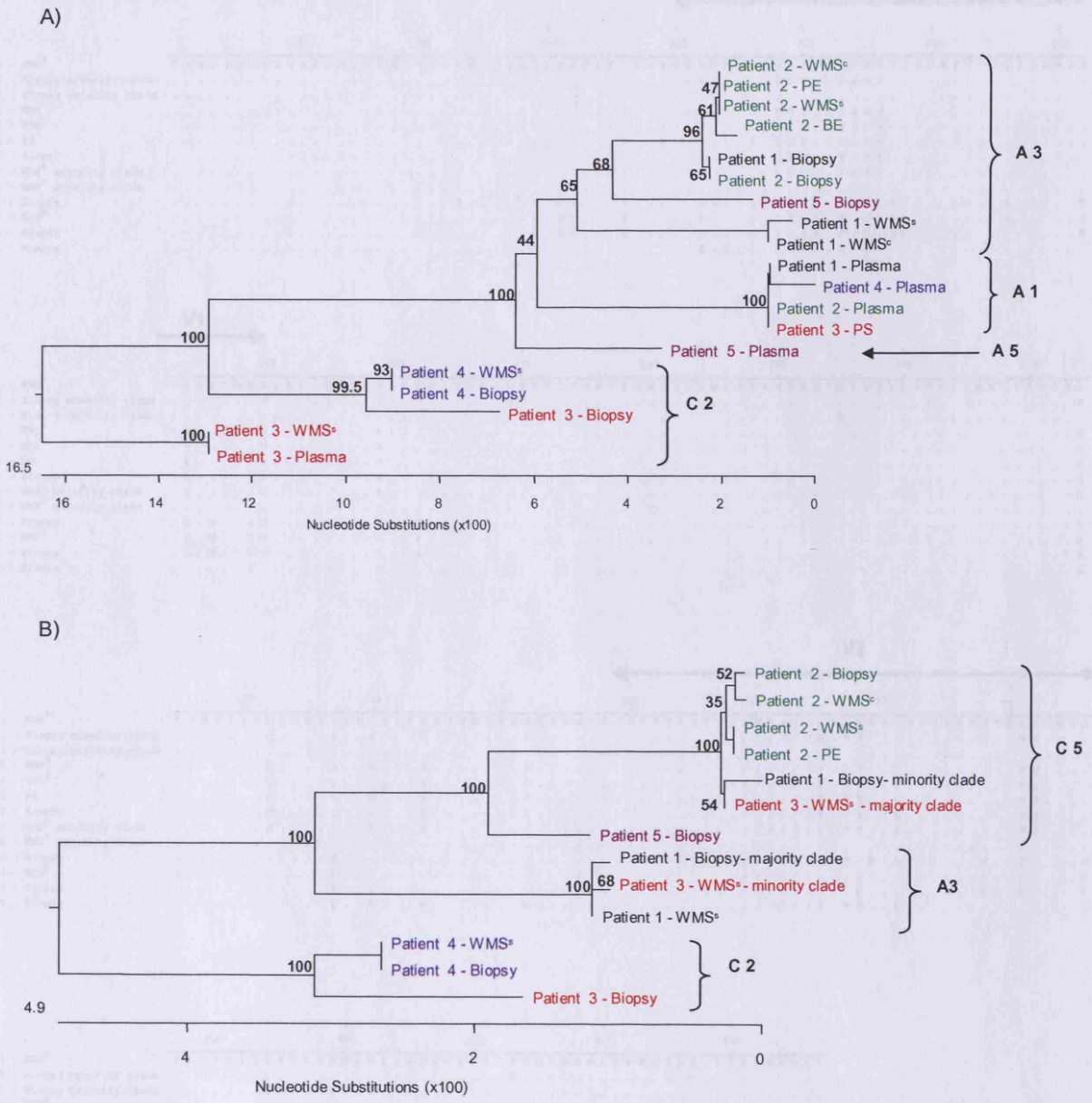


Figure 4.4 RAR-KS intra- and inter-person long K1 amino acid sequence diversity. Dots indicate residues occupying positions aligned to those of Patient 1's WMS^S sequence at top. Slashes denote absence of residues due to nucleotide deletions

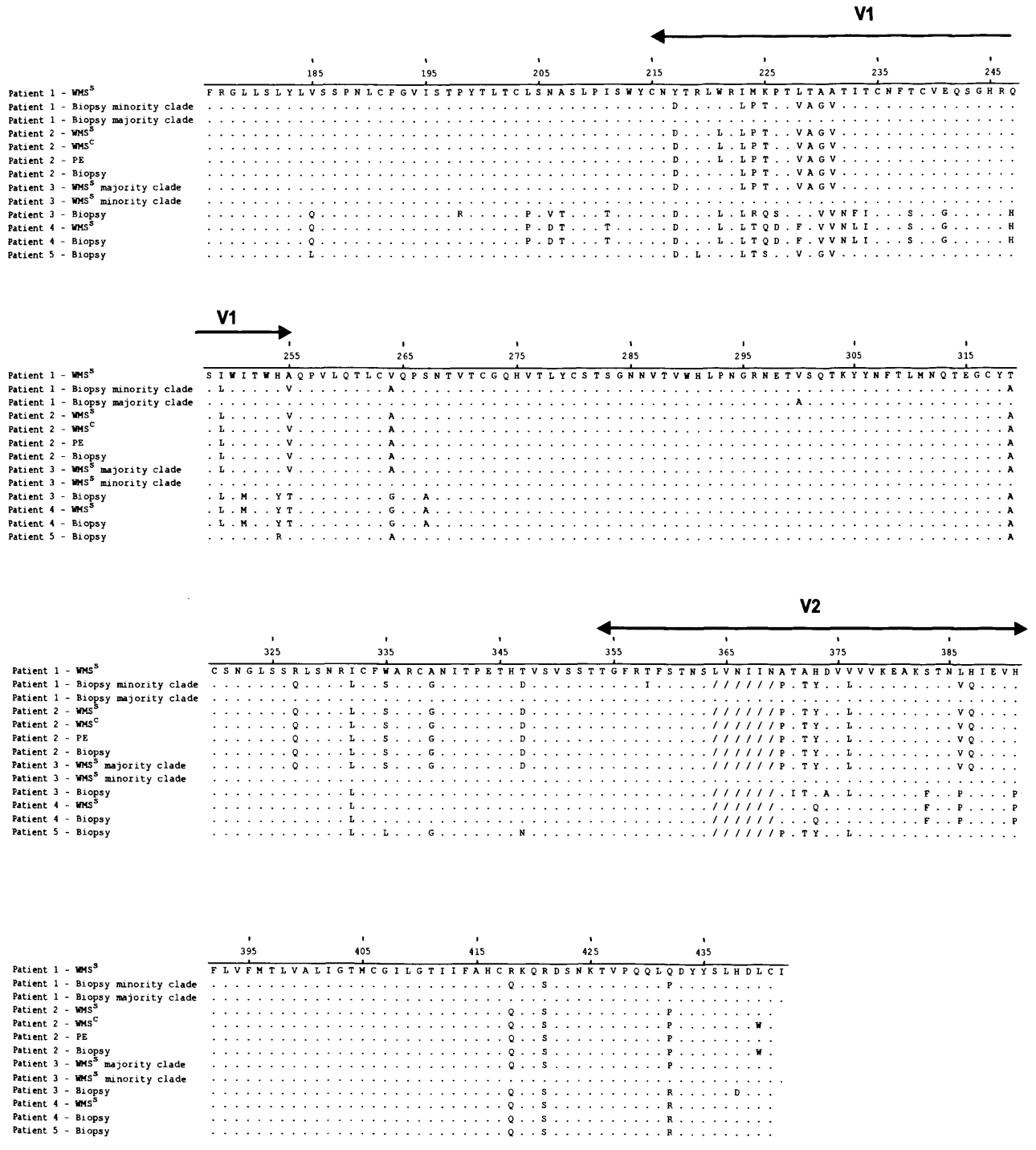


Table 4.3 Linkage analysis of KS330, K1/V1 and longK1 genotypes

Patient	Patient sample	KS330 genotype	K1/V1 genotype	long K1 genotype
1	WMS^s	B	A 3	A 3
	WMS^c	B	A 3	-
	Plasma	-	A 1	-
	Biopsy	B/C	A 3	A 3 & C 5
2	WMS^s	B/C	A 3	C 5
	WMS^c	B/C	A 3	C 5
	BE	B/C	A 3	-
	PE	B/C	A 3	C 5
	Plasma	-	A 1	-
	Biopsy	B/C	A 3	C 5
3	WMS^s	-	C 2	C 5 & A 3
	PS	-	A 1	-
	Plasma	-	C2	-
	Biopsy	B/C	C 2	C 2
4	WMS^s	F	C 2	C 2
	Plasma	-	A 1	-
	Biopsy	F	C 2	C 2
5	Plasma	-	A 5	-
	Biopsy	F	A 3	C 5

WMS^s, whole mouth saliva supernate fraction; WMS^c, whole mouth saliva cellular fraction; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; - , not detected

Table 4.4 The extent of intra-person sequence diversities relative to the KS biopsy sequence

Patient	Region	% nucleotide sequence diversity from KS biopsy sequence					
		Plasma	WMS^s	WMS^c	PS	BE	PE
1	KS330	-	2.5	2.5	-	-	-
	K1/V1	9	7.4	6.9	-	-	-
	long K1	-	5.0*, 0.1$^¥$	-	-	-	-
2	KS330	-	0.6	0.6	-	1.8	1.8
	K1/V1	9	0.5	0.5	-	0.9	0.5
	long K1	-	0.3	0.1	-	-	0.3
3	KS330	-	-	-	-	-	-
	K1/V1	13.3	13.3	-	17.4	-	-
	long K1	-	7.9†, 7.2‡	-	-	-	-
4	KS330	-	0	-	-	-	-
	K1/V1	16.3	0	-	-	-	-
	long K1	-	0	-	-	-	-
5	KS330	-	-	-	-	-	-
	K1/V1	7.4	-	-	-	-	-
	long K1	-	-	-	-	-	-

WMS^s, whole mouth saliva supernate fraction; WMS^c, whole mouth saliva cellular fraction; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; - , not detected
* as compared with the minority clade of Patient 1's biopsy
$^¥$ as compared with the majority clade of Patient 1's biopsy
† as compared with the majority clade of Patient 3's WMS^s
‡ as compared with the minority clade of Patient 3's WMS^s

4.3.2 Intra-person K1/V1 nucleotide sequence differences demonstrated by DGGE

Patient 3's WMS^s and biopsy samples, which showed the greatest saliva-biopsy K1/V1 sequences divergence (Table 4.4), also exhibited, by cloning and DGGE (Figure 4.5), a significant sequence diversity of up to 15% between clones from the two samples, while intra-sample divergence was found to be ≤0.9%. A dendogram generated after sequencing the below DGGE samples is displayed in Figure 4.6.

Figure 4.5 DGGE photograph accommodating K1/V1 DNA amplified from 15 clones generated from supernate fraction of whole mouth saliva (WMS^s) and biopsy samples of patient 3. Arabic numerals represent assigned clone numbers, coinciding with lane positions in the gel.

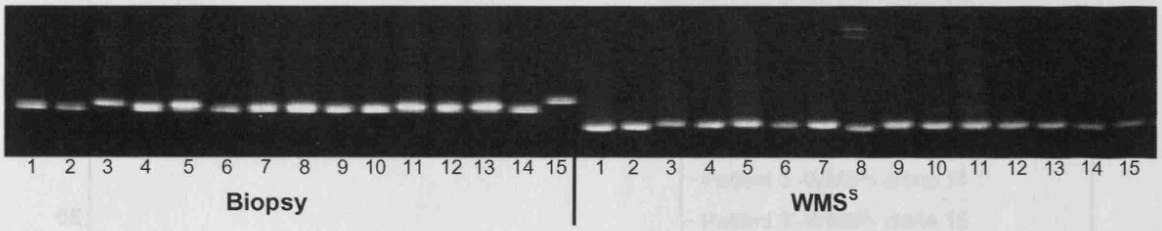
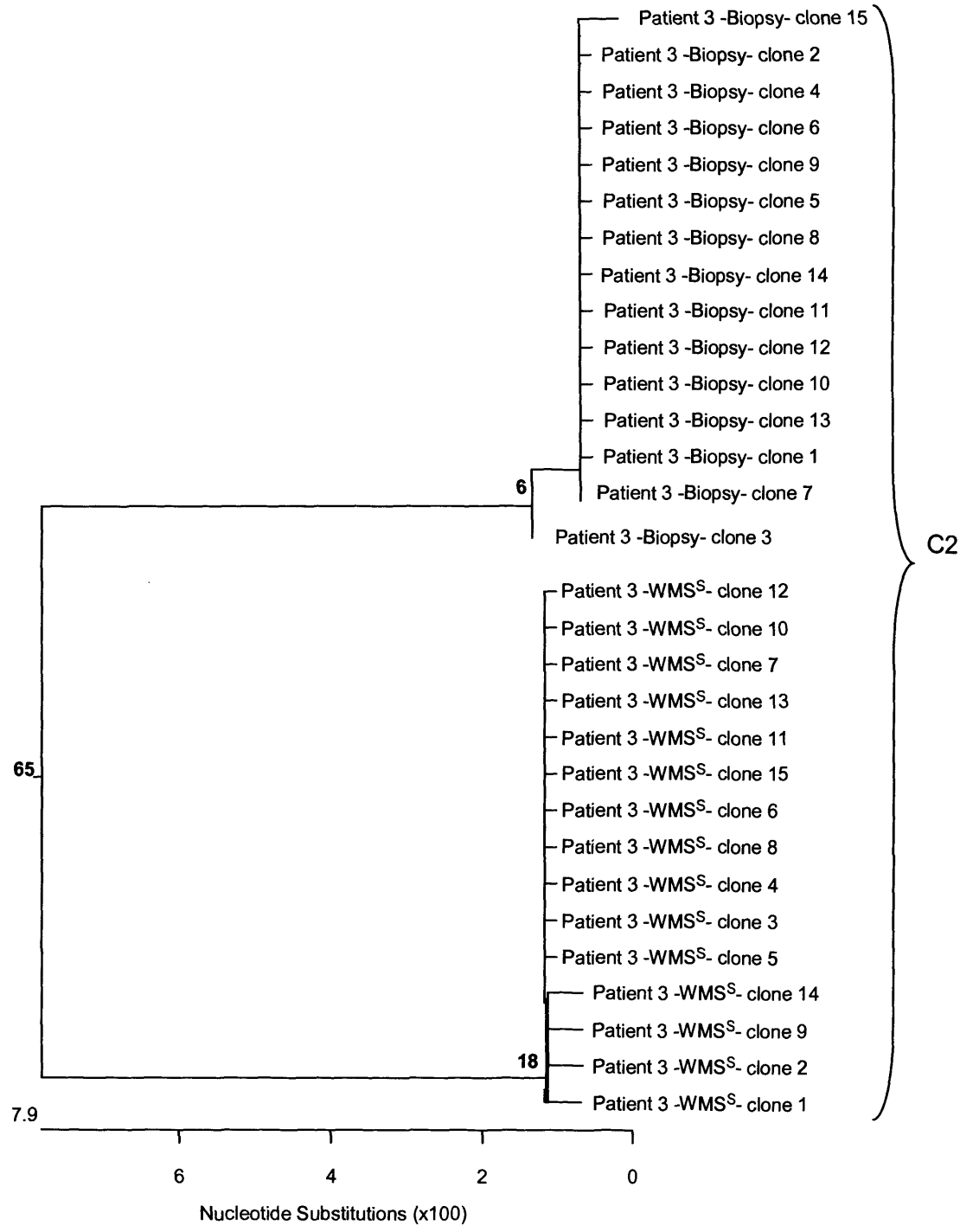


Figure 4.6 Dendrogram showing phylogenetic distribution of K1/V1 consensus derived from clones of patient 3's WMS^{s} and biopsy. Bootstrapping for 1000 replicates is noted as a percentage at major branch points.



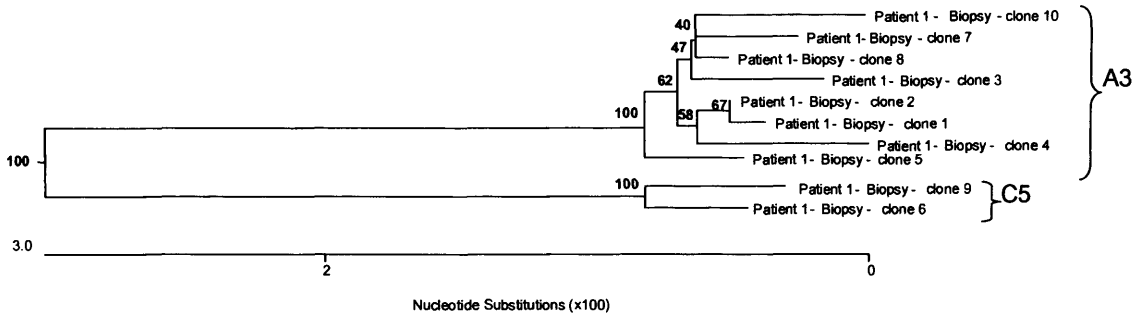
4.3.3 Clonal analysis of inter- and intra-sample long K1 sequence differences by direct sequencing

The sequences of the long K1 clones originating from the saliva and biopsy samples of the 5 individuals belonged to 3 genotypes (A3, C2 & C5). All five patients had confirmed clonal viral sequence variation at the nucleotide and amino acid levels from both the saliva and biopsy samples. Table 4.5 summarizes the range of intra-sample nucleotide divergence between clones for each patient and the genotype designations. For patient 1, cloning studies revealed that sequences of 8 of 10 clones sampled from his biopsy sample belonged to subtype A3 and 2 of them to C5 (Figure 4.7A); the consensus sequence of the A3 clade was 0.1% divergent from the A3 sequence of WMS^s (Fig 4.3B & Fig 4.4). For patient 3, the WMS^s sequences could, by cloning, be resolved into 2 separate clades, 3 of 10 clones belonging to A3, and 7 to C5 (Figure 4.7B); these subtypes were distinct from C2 to which the sequence of his biopsy sample belonged (Fig 4.3B & Fig 4.4). The subgenotypic differences between the intra-sample clades for these two patients were considered to be significant as the divergence between long K1 sequences was > 5%.

High-fidelity K1/V1 PCR of the previously generated clones from patients 1 and 3 yielded K1/V1 DNA to show the different extents of intra-sample diversity in the VR1 region of long K1 colonies (Figure 4.8).

Figure 4.7 Dendrogram showing phylogenetic distribution of long K1 consensus derived from clones of patient 1's biopsy (A) and clones of patient 3's WMS^s (B). Bootstrapping for 1000 replicates is noted as a percentage at major branch points.

(A)



(B)

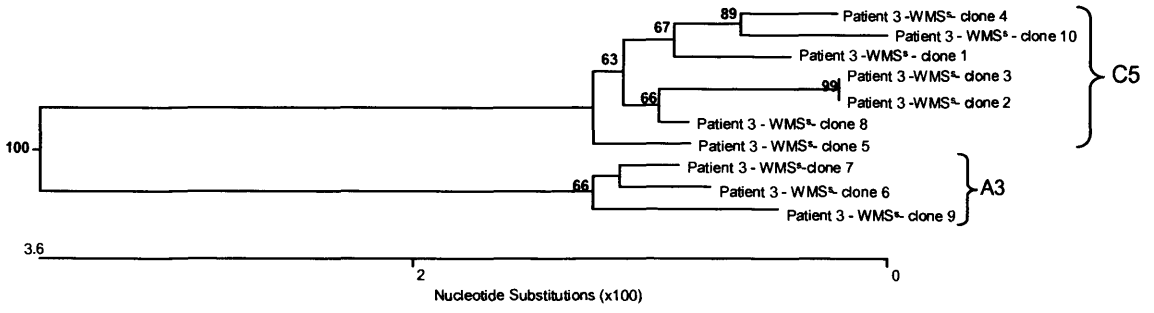
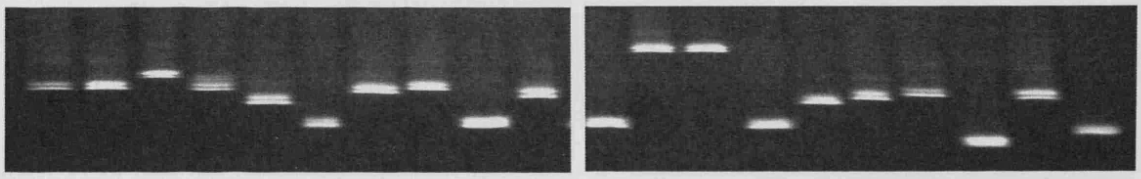


Table 4.5 Range of intra-sample long K1 nucleotide divergence between clones and genotype designation

Patient	No. of clones sequenced		Range of intra-sample nucleotide divergence (%)		No. of clones with minority genotype (genotype) / no. of clones sequenced		Genotype assignment	
	Saliva	Biopsy	Saliva	Biopsy	Saliva	Biopsy	Saliva	Biopsy
1	10	10	0 to 1	0.1 to 5.8	-	2 (C5) /10	A3	A3 + C5
2	10	10	0 to 0.6	0.1 to 0.9	-	-	C5	C5
3	10	11	0 to 6.8	0 to 0.6	3 (A3) /10	-	C5 + A3	C2
4	11	11	0 to 0.8	0 to 0.8	-	-	C2	C2
5	-	10	-	0 to 1.2	-	-	-	C5

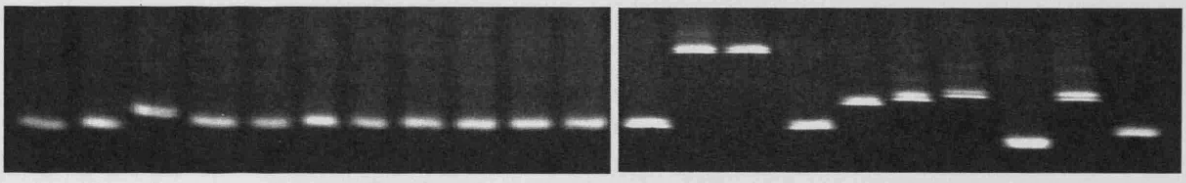
Figure 4.8 Composite of 2 DGGE gel photographs. (A) K1/V1 DNA amplified from 10 clones generated per sample from patients 1 and 3. (B) K1/V1 DNA amplified from 10 clones generated per sample from patient 3. Arabic numerals represent assigned clone numbers, coinciding with lane positions in the gel.

A)



Patient 1 Biopsy **Patient 3 WMS^{s}**

B)



Patient 3 Biopsy **Patient 3 WMS^{s}**

4.3.4 Whole saliva supernate viral load

For 3 patients, viral load estimation in WMS^s was possible (Table 4.2). The load in patient 2 was particularly high. No association was observed between salivary viral load and oral inflammation (as indicated by the plaque, gingival and bleeding oral indices) or between salivary viral load and the dosage of immunosuppressives administered (Table 4.6). However, due to the small sample number, statistical analysis was not possible.

4.3.5 Serological detection of HHV-8

All the plasma samples were reactive for anti-HHV-8 IgG.

Table 4.6 Patients daily dosage of administered immunosuppressives in relation to HHV-8 WMS^s viral loads

Patient	WMS^s viral load copies/mi	Ciclosporin dose (mg)	Prednisolone dose (mg)
1	1238	0	0
2	2198250	0	0
4	3778	150	5

WMS^s, whole mouth saliva supernate fraction

4.4 Discussion

The mean time between kidney transplant and diagnosis of KS in this sample group was 9.7 yr, which is greater than that previously reported in Saudi Arabian renal allograft recipients (al Suleiman *et al.*, 1987; Qunibi *et al.*, 1988; Qunibi *et al.*, 1993). KS affected only the skin in all the 5 patients studied. This is not unusual as the skin is the most common organ involved with KS, followed by the gastrointestinal tract and the lungs, among renal transplant recipients in Saudi Arabia (Qunibi *et al.*, 1993; al Sulaiman and al Khader, 1994). In the present study, the lower limbs were affected in the majority of patients (80%), coinciding with previous reports of skin KS mostly affecting the lower extremities (Moosa, 2005). No oral KS, specifically palatal KS, was reported in this group of patients. This may be due to low replicative activity of HHV-8 in the palatal mucosa (Beyari *et al.*, 2003) or because it had gone undetected during the course of active KS.

The study described in this chapter contributes to the growing body of evidence implicating the oral cavity as a major site of HHV-8 replication and shedding. Earlier studies have found evidence of HHV-8 in oral samples taken from people with or without active KS and those who were or were not co-infected with human immunodeficiency virus (Koelle *et al.*, 1997; Blackbourn *et al.*, 1998; Lampinen *et al.*, 2000; Pauk *et al.*, 2000; Cook *et al.*, 2002b; Beyari *et al.*, 2003; Taylor *et al.*, 2004; Duus *et al.*, 2004; Triantos *et al.*, 2004; Mbulaiteye *et al.*, 2004; Widmer *et al.*, 2006; Casper *et al.*, 2007). HHV-8 DNA was previously found in buccal and palatal exfoliates of Malawian KS patients and their relatives (Cook *et al.*, 2002a; Cook *et al.*, 2002b; Beyari *et al.*, 2003). The present findings show that also for KS-experienced renal allograft recipients the buccal mucosa and palate support HHV-8 persistence. That HHV-8 replicates at these sites is suggested by data from patient 2, whose HHV-8 load in WMS exceeded 2 million genome copies/ml and in whom viral DNA could be detected in both BE and PE samples. The inability to detect HHV-8 DNA in

the BE and PE of some patients does not rule out the possibility that the virus may still replicate in areas of the palatal or buccal mucosa further away from the selected sampling site.

For most of the patients studied, WMS yielded higher PCR amplification rates than other oral samples, likely reflecting HHV-8 shed from other sites, such as tonsils (Koelle *et al.*, 1997; Chagas *et al.*, 2006b) and tongue (Triantos *et al.*, 2004; Widmer *et al.*, 2006). The limited HHV-8 DNA detection rate in saliva collected from the parotid duct supports previous findings indicating that major salivary glands are not important contributors to HHV-8 shedding (Pauk *et al.*, 2000). Gingival crevicular fluid is unlikely to contribute significantly, given that HHV-8 plasma viraemia in the studied patients was not extensive, as suggested by the discordant KS330, K1/V1 and long K1 detection rates in plasma. Semi-quantitative dilution studies showed that the limits of HHV-8 DNA PCR detection for long K1 was 10^3 times less than the limit for K1/V1.

The failure to detect HHV-8 in the saliva of patient 5, who was still undergoing treatment for KS, could be attributed to the fact that HHV-8 shedding in saliva may be episodic in some patients and missed when only single specimens are analyzed (Koelle *et al.*, 1997; Pauk *et al.*, 2000; Laney *et al.*, 2004; Casper *et al.*, 2007). Patients 1 and 4 each had a relatively low saliva HHV-8 load (<50,000 copies/ml). However, HHV-8 viral loads have been reported to be variable and fluctuate over time (Tedeschi *et al.*, 2001).

Previous studies among HIV-infected patients with KS showed that the prevalence of salivary HHV-8 DNA were similar between patients with and without oral KS lesions and that HHV-8 could be detected in saliva from patients with resolved KS (Koelle *et al.*, 1997), an observation consistent with the study patients here who had cutaneous and not oral KS.

HHV-8 DNA was not detected in any blood cellular sample, possibly reflecting the absence of active KS at the time of sampling and the tapering of immunosuppressive drug dosages. Previous reports of the presence of HHV-8 genome in peripheral blood cells reported its detection mainly in people with active KS or at high risk of KS (Purvis *et al.*, 1997; Metaxa-Mariatou *et al.*, 2004), and to a lesser extent in those with regressing iatrogenic KS and in none upon complete remission (Aluigi *et al.*, 1996).

Although previous work found HHV-8 plasma viraemia to be positively associated with active disease (Harrington *et al.*, 1996; Tedeschi *et al.*, 2001; Campbell *et al.*, 2003; Duus *et al.*, 2004), HHV-8 plasma viraemia was found in all the study patients here despite the absence of clinically evident KS in 4 of the 5 patients. These various outcomes in detection rates likely result from different sensitivities of the PCR protocols applied.

The results of previous serologic studies support the view that infection with HHV-8 is nearly universal in patients with non-iatrogenic KS (Lennette *et al.*, 1996; Sitas *et al.*, 1999a; Hbid *et al.*, 2005; Mwakigonja *et al.*, 2007). Studies involving renal allograft recipients with post-transplantation KS found 93% of Saudi (Qunibi *et al.*, 1998) and 100% of Portuguese (Weigert *et al.*, 2004) renal allograft recipients to be seropositive for HHV-8. The finding here, that all 5 renal allograft recipients were seropositive, shows again high concordance between HHV-8 seropositivity and KS.

After thorough intra-oral examination and data analysis we were unable to find an association between inflammation in the oral cavity and HHV-8 shedding, contrary to what was reported elsewhere (Casper *et al.*, 2004). However, due to the small sample size, this interpretation should be treated with caution.

The extreme polymorphism of ORF K1 was exploited to investigate the genomic diversity of HHV-8 more comprehensively (Hayward and Zong, 2007). The genotypes of HHV-8 (A, B, C and D) differ in this ORF by sequence divergences that vary between 5% and 30% at the amino acid level, with A and C being the most related. Genotyping and subtyping are based on the extent of amino acid sequence differences and the presence of specific deletions or duplications. In the study reported in this chapter, K1/V1 and long K1 segments from ORF K1 were amplified. The K1/V1 fragment, being shorter, confers higher sensitivity to PCR detection of the HHV-8 genome, but limits the information on the extent of HHV-8 genomic sequence polymorphism. The long K1 segment, by contrast, lends lesser sensitivity to PCR detection but the amplicons, being >3 times longer than K1/V1, potentially allow HHV-8 strains to be better differentiated. Particularly as it encompasses the VR2 region, long K1 enables sharper discrimination between A and C genotypes. Thus, long K1 sequencing led to the assignment of sequences originating from patient 5's biopsy as well as patient 2's biopsy, WMS and PE to C5 (Figure 4.3B) on account of the 15-bp deletion in the VR2 region (Figure 4.4), whereas K1/V1 sequencing assigned these samples to an A genotype (Figure 4.3A). However, not all samples assigned an A genotype by K1/V1 sequencing, were assigned to C5 following long K1 sequencing (Table 4.3). Therefore, when for such samples, K1/V1 sequence is available alone, the genotype should preferably be referred to as A3/C5, especially since the C5 variant is thought to be a hybrid between A and C genotypes (Zong *et al.*, 1999). Noteworthy is that the C5 variant has been previously identified by Zong *et al.* (1999) in KS samples from Saudi Arabian renal transplant recipients.

Direct sequencing studies, however, have an important limitation in that they are unable to produce consensus sequence data that will allow sequences of minority variants to be identified. Whereas, the high HHV-8 genome load (Tedeschi *et al.*, 2001) favours the generation of sequences reflecting the dominant variants.

Accordingly, the combined DGGE screening-nucleotide sequencing protocol was applied to K1/V1 amplicons generated from clonal inserts. To reduce the degree to which PCR induces nucleotide misincorporation, which may lead to false representations of natural inter- or intra- sample sequence polymorphism, high-fidelity PCR was applied to sample extracts. By applying this combined DGGE-sequencing protocol, it could be shown that patient 3's saliva and biopsy samples exhibited a significant sequence diversity of up to 15% between the saliva and biopsy clones. This is slightly higher than the 13.3% detected by direct sequencing of K1/V1 PCR products.

When clones of long K1 sequences were characterised, 2 populations of HHV-8 variants in the KS biopsy sample of patient 1 and in the WMS^s of patient 3 could be identified. The populations of HHV-8 variants in the biopsy sample of patient 1 implies concurrent HHV-8 infection at the lesional level and does not support the view that KS is monoclonal (Rabkin *et al.*, 1997; Judde *et al.*, 2000). In each of the samples, the clade comprising minority sequences was genotypically distinct from the majority clade. Further evidence substantiating the phenomenon of multiple HHV-8 infection comes from the divergences in K1/V1 sequences observed in the oral samples of patient 3. These findings point to intra-compartmental (oral) carriage of multiple HHV-8 strains.

Inter-compartmental multiple HHV-8 infection is revealed from the comparison of sequences originating from oral, plasma and biopsy samples. Of the long K1 sequences recovered from 4 saliva-biopsy sample pairs, complete sequence identity was observed in one patient only (patient 4). In the other 3 patients, the maximal intra-person divergences in sequences between an oral sample and biopsy were 5% for patient 1, 0.3% for patient 2 and 7.9% for patient 3 (Table 4.4). While the divergences in patient 2 may be insubstantial and attributable to PCR-generated

mutation, those in patients 1 and 3 were relatively wide, and genotypically disparate. Another set of data implicating multiple inter-compartmental HHV-8 carriage arises from the wide divergences in K1/V1 amino acid sequences between plasma and biopsy sequences of all 5 patients, ranging from 7.4% to 16.3% at the nucleotide (bp) level.

The findings from this study are supported by other reports (Gao *et al.*, 1999; Beyari *et al.*, 2003) but contrast with some studies in individuals with (Meng *et al.*, 2001; Stebbing *et al.*, 2001; Zong *et al.*, 2002) and without (Mbulaiteye *et al.*, 2006) KS, which have not revealed evidence for intra-person variation in HHV-8 sub-genomic sequences. The paucity in the number of reports of mixed HHV-8 infection may be due to a combination of factors, as described in Chapter 3. The design of the current study precludes resolution of whether concurrent HHV-8 infections in the patients resulted from co-infection or super-infection, of the transplanted kidney as source of infection (Parravicini *et al.*, 1997; Regamey *et al.*, 1998), or of the extent to which immunosuppression predisposed the recipients to reactivation or future HHV-8 infection.

Because HHV-8 genomic sequences carried in biopsy, plasma and oral samples from these 5 patients with KS could be either genotype, conclusions could not be drawn about the selective tropism of HHV-8 strains. Current evidence from other studies suggests that HHV-8 exhibits broad tropism (Dupin *et al.*, 1999; Pauk *et al.*, 2000; Blackbourn *et al.*, 2000).

This study has therefore identified the oral cavity as a major site of HHV-8 shedding in KS-experienced renal allograft recipients, and suggests that their saliva has greater potential than blood to spread HHV-8. Further, such patients may be infected by multiple HHV-8 strains, implying their poor immunity from past infection.

Chapter 5

Organ-related HHV-8 Transmission and Reactivation in Renal Transplant Recipients

5.1 Introduction

Post-transplant KS may be caused by two possible mechanisms: HHV-8 reactivation in patients who were infected before the graft; and HHV-8 neo-infection either by blood products or from allogeneic transplant tissue. Studies from various groups, which generate findings based on serology and molecular tracking of infection (summarized in Table 5.1) have shown that organ-related transmission of HHV-8 is more common than previously thought. Primary infection after transplantation has been suggested as posing a greater risk of complications than reactivation of pre-existing virus (Sarid *et al.*, 2001; Marcelin *et al.*, 2004), and the risk of KS can be exceedingly high when the organ donor and recipient are both HHV-8-positive (Parravicini *et al.*, 1997; Bergallo *et al.*, 2007). Therefore the distinction between HHV-8 reactivation and primary infection may be important for predicting outcome in regard to KS development. Accordingly, systematic screening for HHV-8 antibodies in recipients and organ donors and screening of grafts for HHV-8 have been advocated (Milliancourt *et al.*, 2001; Marcelin *et al.*, 2007).

This chapter reports an investigation into the geno- and sero-prevalence of HHV-8 in a group of patients awaiting kidney transplantation and in their donors. Furthermore, the potential for a HHV-8 transmission via organ transplantation and the correlation between HHV-8 infection and the development of KS was also investigated.

Table 5.1 Solid organ transplant recipients developing KS

Reference	Type of transplant	Country	No. of recipients HHV-8 positive before transplantation and developing KS / recipients developing KS (%)	No. of recipients HHV-8 sero-converting after transplantation and developing KS / recipients developing KS (%)	No. of HHV-8 geno- or sero-positive corresponding donors / donors to recipients developing KS (%)
Parravicini *et al.* (1997)	Heart and kidney	Italy	10 / 11 (91%)	1 / 11 (9%)	4* / 11 (36.4%)
Regamey *et al.* (1998)	Kidney	Switzerland	0 / 2 (0%)	2 / 2 (100%)	NA
Sachsenberg *et al.* (1999)†	Lung	Switzerland	1 / 1 (100%)	0 / 1 (0%)	0 / 1 (0%)
Frances *et al.* (2000)	Kidney	France	9 / 9 (100%)	0 / 9 (0%)	NA
Luppi *et al.* (2000)†	Kidney	Italy	0 / 2 (0%)	2 / 2 (100%)	1*/ 1 (100%)
Andreoni *et al.* (2001)	Kidney and liver	Italy	3 / 4 (75%)	1 / 4 (25%)	NA
Cattani *et al.* (2001)	Kidney	Italy	6 / 7 (86%)	1 / 7 (14%)	NA
Kapelushnik *et al.* (2001) †	Kidney	Israel	0 / 1 (0%)	1 / 1 (100%)	1* / 1 (100%)
Munoz *et al.* (2002)	Kidney, liver and heart	Spain	NA	3 / 7 (43%)	NA
Emond *et al.* (2002)	Heart	France	1 / 1 (100%)	0 / 1 (0%)	NA
Collart *et al.* (2004)†	Heart	France	0 / 1 (0%)	1 / 1 (100%)	1* / 1 (100%)
Marcelin *et al.* (2004)	Liver	France	0 / 2 (0%)	2 / 2 (100%)	2* / 2 (100%)
Becuwe *et al.* (2005)	Kidney, Kidney-pancreas, Liver and Heart	France	16 / 20 (80%)	2 / 20 (20%)	NA
Bergallo *et al.* (2007)	Kidney	Italy	1 / 1 (100%)	0 / 1 (0%)	1 / 1 (100%)

*, including donors to the seroconverting recipients; †, case report; NA, not available

5.2 Patients, Material and Methods

5.2.1 Study population

The study sample included 6 CRF patients undergoing kidney transplant (CRF-tr), as well as 6 renal allograft donors (RADs) and 3 relatives of renal allograft recipients (RRARs). All renal transplant surgeries were conducted at the Armed Forces Hospital in Riyadh, Saudi Arabia during the months of March, April and May, 2004. The mean age of the renal allograft recipients was 35 yr while the mean age of the allograft donors was 28 yr. In both donor and recipient groups, the male:female ratio was 5:1, while all RRARs were male. Four out of the six CRF-tr patients received renal allografts from live related donors, while the remaining two received allografts from cadavers. Five out of the six CRF-tr patients were Saudi Arabian (3 from Jizan and 2 from Riyadh) and one was Jiboutian. The RADs were of four different nationalities (Table 5.2). Serum from the CRF-tr patients, RADs and RRARs was tested, prior to kidney transplantation, for various infectious agents by the hospital's haematology department (Table 5.3). After 3 months post-transplantation, one patient, CRF-tr 5, the only non-Saudi CRF-tr amongst the participants, travelled back to his home country (Jibouti), where his medical care was continued.

Table 5.2 Demographic and clinical characteristics of renal allograft recipients and donors at transplantation

		CRF-tr			RAD				
CRF-tr / RAD pair no.	Date of transplant	Gender	Age* (years)	Nationality	Gender	Age* (years)	Nature of allograft	Relation to recipient	Nationality
1	29 - 3 - 04	M	30	Saudi	M	32	live	brother	Saudi
2	17 - 3 - 04	F	36	Saudi	M	30	cadaver	-	Pakistani
3	30 - 3 - 04	M	38	Saudi	M	30	cadaver	-	Indian
4	3 - 4 - 04	M	33	Saudi	F	21	live	sister	Saudi
5	7 - 4 - 04	M	37	Jiboutian	M	33	live	brother	Jiboutian
6	8 - 5 - 04	M	34	Saudi	M	24	live	brother	Saudi

CRF-tr, patients with chronic renal failure undergoing kidney transplant; RAD, renal allograft donors; *, age at time of transplant

Table 5.3 Results of pre-transplant antibody testing for various infectious agents

CRF-tr / RAD / RARR Groups		HBV	HCV	HIV	HSV	VZV	EBV	CMV
1	CRF-tr	neg	neg	neg	NT	NT	pos*	1:128
	RAD	neg	neg	neg	1:32	NT	NT	1:32
	RARR-1	neg	neg	neg	NT	NT	NT	NT
	RARR-2	neg	neg	neg	NT	NT	NT	NT
2	CRF-tr	neg	neg	neg	1:64	1:8	NT	1:128
	RAD	neg	neg	neg	NT	NT	NT	NT
3	CRF-tr	neg	neg	neg	NT	NT	NT	NT
	RAD	pos†	neg	neg	NT	NT	NT	neg
4	CRF-tr	neg	pos	neg	NT	NT	NT	neg
	RAD	neg	neg	neg	1:64	NT	NT	1:128
	RARR	neg	neg	neg	NT	NT	NT	NT
5	CRF-tr	neg	neg	neg	1:64	NT	NT	1:128
	RAD	neg	neg	neg	1:16	NT	NT	NT
6	CRF-tr	neg	neg	neg	1:32	NT	NT	1:8
	RAD	neg	neg	neg	1:32	NT	NT	1:16

CRF-tr, patient with chronic renal failure undergoing kidney transplant; RAD, renal allograft donor; RARR, relative of renal allograft recipient;
*, EBV test result obtained from a different testing centre; †, Hepatitis B core antibody positive only

5.2.2 Sample collection

After a standardized interview and oral examination, as detailed in Table 2.1 and Appendix 1, blood samples were obtained from the RRARs while matched oral and blood samples were obtained from both the CRF-tr and the RAD groups, as described in Sections 2.2.1, 2.2.2 and 2.2.3, with the exception of the two cadaver donors. The oral samples were collected immediately after the blood samples were obtained. Samples from both the live RAD and RRAR groups were obtained pre-transplantion only, while samples from CRF-tr patients were obtained both pre- and post-transplantion; at weeks 1, 2, 4, 6 and 8 from all six CRF-tr patients and at month 8 from 5 of the 6 CRF-tr patients.

5.2.3 Sample processing

After separation of plasma from the blood, the CD45+ cell subset was immunomagnetically fractionated from the blood samples of the CRF-tr, live RAD and RRAR groups as described in Sections 2.2.1.1 and 2.2.1.2. In addition, CD31+, CD19+, CD14+ and CD2+ cell subsets were immunomagnetically fractionated from the CRF-tr and live RAD blood samples. The cellular and supernate fractions of WMS (WMS^c and WMS^s, respectively) were separated by low-speed centrifugation. BE and PE were similarly pelleted. Following aspiration of the supernate, WMS^c, BE and PE were resuspended in 1 ml of PBS, and stored at -20°C until required. DNA was extracted from blood cell subsets and oral samples as described in Section 2.3.2. The presence of DNA in each extract was verified by amplifying a fragment of the β-globin gene as described in Section 2.3.3.

5.2.4 Sequencing analysis of DNA amplified from KS330 and K1

The 211-bp KS330 was amplified from sample extracts by use of nested PCR (Section 2.3.4). The 247-bp K1/V1 was amplified from sample extracts by nested PCR (Section 2.3.5.1). In addition, all HHV-8 K1/V1- or KS330-DNA-positive samples

were amplified for long K1 by nested PCR (Section 2.3.5.2). Extracts were tested at least twice for KS330, K1/V1 and long K1 DNA, and considered positive when PCR repeatedly yielded products. PCR products were sequenced, raw DNA sequence data were analyzed and phylogenetic analyses were performed as detailed in Sections 2.3.10 & 2.3.11.

5.2.5 Quantitative PCR

All WMS[s] and PS extracts, showing positive results for K1/V1 or KS330 DNA, or both, were sent to the National Centre for Infectious Diseases, Centres for Disease Control and Prevention, Atlanta, GA, where a quantitative, fluorescence-based, real-time PCR was applied (Section 2.4).

5.2.6 Anti HHV-8 IgG detection

The Advanced Biotechnologies ELISA was applied to all plasma samples (Section 2.5).

5.2.7 Clinical progress

The clinical progress and laboratory investigations of all CRF-tr patients, both pre- and post-transplantion, were monitored and recorded.

5.3 Results

5.3.1 HHV-8 DNA detection

5.3.1.1 In blood

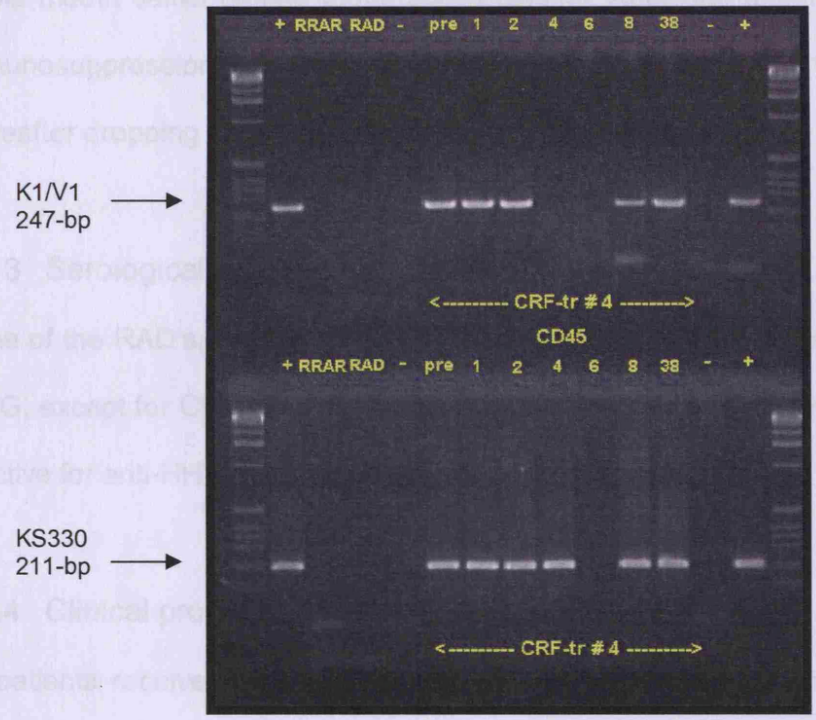
HHV-8 DNA was not detected in the peripheral leucocyte fractions of live RADs or RRARs. It was detected in the peripheral leucocyte fractions of only one CRF-tr patient (CRF-tr 4) pre- and post-transplant (discussed in Chapter 6). Figure 5.1 is a gel electrophoresis image showing HHV-8 DNA in CD45+ cells obtained from CRF-tr 4.

5.3.1.2 In oral samples

HHV-8 DNA was not detected in oral samples of the live RADs. It was detected in the WMS^s (Figure 5.1) and other oral samples (discussed in Chapter 6) of one (CRF-tr 4) out of the six CRF-tr patients.

Figure 5.1 Gel eletcrophoretic images of (A) CD45+ cells and (B) WMS^S of CRF-tr/RAD/RRAR 4.

(A)



5.3.2 Whole saliva supernate viral load

WMS^s HHV-8 viral load was determined for CRF-tr 4. Prior to transplantation, his whole mouth saliva carried 2,606,250 copies/ml. After one month of post-transplant immunosuppression, the HHV-8 WMS^s load increased to 4,106,500 copies/ml, thereafter dropping significantly (discussed in Chapter 6).

5.3.3 Serological detection of HHV-8

None of the RAD's, RRAR's or CRF-tr's plasma samples were reactive for anti-HHV-8 IgG, except for CRF-tr 4's, who's pre- and post-transplant plasma samples were all reactive for anti-HHV-8 IgG.

5.3.4 Clinical progress

All patients received oral valaciclovir post-transplantation at various doses and for various periods of time. CRF-tr 1 developed chicken pox at 4 months post-transplant and CRF-tr 3 developed herpes zoster at 5 months post-transplant. These infections were treated by intravenous aciclovir and famciclovir respectively.

Twenty-six wk following allograft receipt, CRF-tr 4 developed cutaneous KS at the upper trunk, arms and legs, which was later confirmed histologically. KS affecting the stomach and colon was later diagnosed 14 and 15 months post-transplant, respectively (discussed in Chapter 6).

5.4 Discussion

This chapter describes an investigation into HHV-8 reactivation and organ-related transmission in a prospectively studied group of CRF patients undergoing kidney transplantation. The work was accomplished through the examination of oral and blood samples for HHV-8 geno- and sero- positivity, from both the allograft recipients and their corresponding donors.

Pre-transplant prevalence of HHV-8 antibodies among graft recipients was 1/6 (16.7%), which is identical to the seroprevalence we previously detected in Saudi CRF patients (Chapter 3), but less than that detected (29%) by Qunibi *et al.* (1998) and greater than that detected (7%) by Almuneef *et al.* (2001) in ESRD patients. Unlike most studies reporting seroprevalences among donors and recipients which found similar rates to those among the general population of the country considered (Cattani *et al.*, 2001), an increased HHV-8 seroprevalence in Saudi CRF patients undergoing renal allograft transplant when compared with Saudi blood donors (0.6%) is observed.

Previous reports from Saudi Arabia have found the incidence of KS among 350 (al Suleiman *et al.*, 1987) 263 (Qunibi *et al.*, 1988) and 730 (al Sulaiman and al Khader, 1994) renal allograft recipients, who were followed for more than three months, to be 3.4%, 5.3% and 4.7%, respectively. In addition, Alzahrani *et al.* found a very high HHV-8 seropositivity rate (70%) in an Arab family affected with KS. In the present small sample study, the overall incidence of KS after transplantation was 16.7%.

All seronegative recipients with seronegative donors did not seroconvert at any time during the follow-period, coinciding with similar findings in Italy (Andreoni *et al.*, 2001). However, for one patient, the latest sample collected was at 8 wk post-transplant. Therefore, the possibility of seroconversion occurring at a later date

cannot be excluded, although it is unlikely.

In the case of the two renal allograft recipients (CRF-tr 1 and CRF-tr 3) who developed post-transplant chickenpox and herpes zoster respectively, it cannot be known whether these infections were primary or were transmitted via the transplanted graft, as VZV screening was not done for both these allograft recipients or their corresponding donors.

Routine hepatitis B assessment of organ donors usually includes testing for HBsAg, anti-HBs and anti-HBc. Although HBsAg positivity excludes from organ donation, anti-HBc-positive donors may be used under certain life-threatening circumstances, even if their infectivity is established. Nonetheless these grafts were found to be suitable either to effectively vaccinated recipients or to those who are given HBIG to prevent HBV recurrence (Roque-Afonso *et al.*, 2002).

There are only a few case-control studies of solid organ transplant recipients with and without KS, matched to their respective donors investigating the risk of HHV-8 transmission from donor grafts (Parravicini *et al.*, 1997; Marcelin *et al.*, 2004; Bergallo *et al.*, 2007). Most other studies have evaluated the seroprevalence of HHV-8 in groups awaiting heart (Emond *et al.*, 2002), kidney (Regamey *et al.*, 1998; Frances *et al.*, 2000; Cattani *et al.*, 2001; Andreoni *et al.*, 2001) or liver (Andreoni *et al.*, 2001) transplants. However, similar studies are lacking in Middle Eastern countries such as Saudi Arabia, where precise estimates of HHV-8 seroprevalence in the organ donor and recipient populations are needed.

Most post-transplant KS develops as a result of viral reactivation, whereas more than 75% of transplant recipients developing KS have been reported to be sero-positive for HHV-8 before undergoing transplantation. In an Italian retrospective study, 10 of

11 (91%) organ recipients who developed KS were HHV-8 seropositve before transplantation (Parravicini *et al.*, 1997). This finding was later supported by other Italian studies, where 6 of 7 (86%) (Cattani *et al.*, 2001) and 3 of 4 (75%) (Andreoni *et al.*, 2001) organ recipients who developed KS were also found to be seropositive before transplantation. In France, a country of low HHV-8 endemicity, no KS was observed in renal allograft recipients without anti-HHV-8 antibodies at the time of transplantation (Frances *et al.*, 2000). Additionally, a more recent French study, reported 16 of 20 (80%) organ recipients developing KS as being seropositive before transplantation (Becuwe *et al.*, 2005).

The first report suggesting *de novo* infection from transplantation involved a kidney transplant patient in Italy who was HHV-8-seronegative before transplantation and seroconverted 13 months after transplantation with onset of Castleman disease (Parravicini *et al.*, 1997). Cases of HHV-8 seroconversions after kidney transplantation resulting in KS development have been identified in Italy (Cattani *et al.*, 2001; Andreoni *et al.*, 2001), Israel (Kapelushnik *et al.*, 2001) and Spain (Munoz *et al.*, 2002). In a cohort of 220 transplant recipients in Switzerland, KS developed within 26 months in two of 25 patients who had developed a primary HHV-8 infection after kidney transplantation (Regamey *et al.*, 1998). Similarly, cases of HHV-8 seroconversion and KS development after heart (Collart *et al.*, 2004) and liver transplantation (Marcelin *et al.*, 2004) have been reported. These studies suggested that transmission of HHV-8 can occur by the organ allograft. Moreover, a primary HHV-8 infection has been described in two patients after both received kidneys from a seropositive cadaver donor. One of the patients developed disseminated KS, and the other developed an acute syndrome characterized by fever with plasmacytosis followed by bone marrow failure and ultimately died of renal and cardiac failure (Luppi *et al.*, 2000b). Sex and genetic markers specific to the donor have been detected in neoplastic HHV-8-infected cells in KS lesions isolated from renal transplant

recipients, suggesting that not only HHV-8 but also KS progenitor cells may be seeded after solid organ transplantation, survive in the recipients, and undergo neoplastic transformation and progression (Barozzi *et al.*, 2003).

The results reported in this chapter do not suggest HHV-8 transmission via organ transplantation, as none of the renal allograft donors were geno- or sero-positive for HHV-8. In addition, the only seropositive allograft recipient was both geno- and sero-positive for HHV-8 prior to transplantation. Therefore, the suggestion that HHV-8 transmission in regions where the virus is not endemic occurs via the transplanted organ (Regamey *et al.*, 1998; Marcelin *et al.*, 2004) cannot be applied to the sample group studied here, in whom other factors could have contributed to KS development. However, in the view of the small number of participants, no definitive conclusion regarding the most likely cause of KS in the Saudi renal allograft recipients may be drawn.

Knowledge of the HHV-8 infection status of the graft donor and recipient can help in identifying individuals at high risk for post-transplantation KS, early detection and management of KS. This small case control study is the first prospective study conducted in Saudi Arabia evaluating the prevalence of HHV-8 in organ donors and corresponding recipients; hence it is described in a separate (this) chapter. The findings, though not definitive, stress the need to proceed with larger-scale studies of this nature, including screening cadaver donors for HHV-8, to determine the risk of post-transplantation KS, its relationship to HHV-8 reactivation or organ-related transmission, and the morbidity and mortality due to KS.

Chapter 6

Persistent HHV- 8 Oral and Blood Shedding Prior to Onset of KS in a Renal Transplant Recipient

6.1 Introduction

Previous studies have shown that HHV-8 infection, identified by the presence of HHV-8 DNA sequences in PBMCs or of serum antibodies to HHV-8, precedes and is predictive of the subsequent onset of KS (Whitby *et al.*, 1995; Gao *et al.*, 1996a; Gao *et al.*, 1996b; Moore *et al.*, 1996c; Whitby *et al.*, 1998; Pozo *et al.*, 2000; Garcia-Astudillo and Leyva-Cobian, 2006). However, not all pre-transplantation HHV-8 seropositive patients develop KS even when they are undergoing immunosuppression (Parravicini *et al.*, 1997; Frances *et al.*, 2000; Cattani *et al.*, 2001; Bergallo *et al.*, 2007). In addition, it is not known if HHV-8 shedding from the oral cavity, an important site of shedding (Teo, 2006), can precede, and potentially predict development of KS in people given immunosuppressive drugs.

This chapter describes an evaluation of the extent of oral and blood HHV-8 shedding in a prospectively-studied individual who developed KS 6 months after receiving a renal allograft. Included in the study is an evaluation of the cellular distribution of HHV-8 DNA detection in PBMC sub-populations, multiple HHV-8 infection and the effects of immunosuppressive drugs, antiviral drugs and oral health on salivary HHV-8 viral load.

6.2 Patient, Material and Methods

6.2.1 Patient and sample collection

Matched oral and blood samples were obtained from a 33-yr-old Saudi male who was undergoing renal allograft transplantation at the Armed Forces Hospital in Riyadh, Saudi Arabia in April 2004. Samples were obtained pre-transplant and up to 8 months post transplant. This patient is identical to CRF-tr 4 of Chapter 5. At each visit, a standardized interview and oral examination, as detailed in Table 2.1 and Appendix 1, were conducted. This was followed by the collection of body fluid samples as described in Sections 2.2.1, 2.2.2 and 2.2.3. The oral samples were collected

immediately after the blood samples were obtained.

6.2.2 Sample processing

After separation of plasma from the blood, the CD45+, CD31+, CD19+, CD14+ and CD2+ cell subsets were immunomagnetically fractionated as described in Sections 2.2.1.1 & 2.2.1.2. The cellular and supernate fractions of WMS (WMS^c and WMS^s, respectively) were separated by low-speed centrifugation. BE and PE were similarly pelleted. Following aspiration of the supernate, WMS^c, BE and PE were resuspended in 1 ml of PBS, and stored at -20°C until required. DNA was extracted from plasma, blood cell subsets and oral samples as described in Section 2.3.2. The presence of DNA in each extract was verified by amplifying a fragment of the β-globin gene as described in Section 2.3.3.

6.2.3 Sequencing analysis of DNA amplified from KS330 and K1

The 211-bp KS330 was amplified from sample extracts by use of nested PCR (Section 2.3.4). The 247-bp K1/V1 was amplified from sample extracts by nested PCR (Section 2.3.5.1). In addition, all HHV-8 KS330 or K1/V1 DNA positive samples were amplified for long K1 by nested PCR (Section 2.3.5.2). Extracts were tested at least twice for KS330, K1/V1 and long K1 DNA, and considered positive when PCR repeatedly yielded products. PCR products were sequenced, raw DNA sequence data were analyzed and phylogenetic analyses were performed as detailed in Sections 2.3.10 and 2.3.11.

6.2.4 Combined DGGE screening-nucleotide sequencing to reveal inter- and intra-sample K1/V1 sequence differences

Samples which concordantly amplified positively for KS330, K1/V1 and long K1 DNA and showed a significant divergence in their K1/V1 sequences were subjected again to nested PCR by use of the EXPAND High Fidelity PCR System, as described in

Section 2.6.1. The PCR products were then purified directly from the second-round PCR product (Section 2.3.8) and clones were generated (Section 2.3.9). From each amplificate, 22 colonies were randomly picked, and each colony subjected to another round of PCR using a clamping primer as described in Section 2.6.2. The PCR products from 14 selected colonies were subjected to DGGE (Beyari *et al.*, 2003) as detailed in Sections 2.6.3, 2.6.4 and 2.6.5, followed by nucleotide sequencing (Section 2.3.10). Raw DNA sequence data were analyzed using Seqman software (DNAstar) (Section 2.3.11).

6.2.5 Quantitative PCR

All WMS^s and PS extracts, showing positive results for K1/V1 or ORF 26 DNA or both, were sent to the National Centre for Infectious Diseases, Centres for Disease Control and Prevention, Atlanta, GA, where a quantitative, fluorescence-based, real-time PCR was applied (Section 2.4).

6.2.6 Anti HHV-8 IgG detection

The Advanced Biotechnologies ELISA (Section 2.5) was applied to all plasma samples collected pre and post transplantion.

6.3 Results

6.3.1 HHV-8 DNA detection

6.3.1.1 In blood

HHV-8 was detected in the patient's PBMCs both pre- and post-transplant. However, plasma viraemia was not observed until 9 months post-transplant. Seven blood samples (7/42;17%) were concordantly positive for KS330 and K1/V1 (CD19+ cells: 1; CD31+ cells: 1; and CD45+ cells: 5), 3 samples (3/42; 7%) were positive for K1/V1 only (CD2+ cells: 1; CD19+ cells: 1; and CD31+ cells: 1), and 4 samples (4/42; 9.5%) for KS330 alone (CD19+ cells: 2; CD45+ cells: 1; and plasma:1) (Table 6.1). Seven blood samples (7/14; 50%) were also positive for long K1 DNA, six of which were concordantly positive for KS330 and K1/V1 (Table 6.2).

6.3.1.2 In oral samples

HHV-8 was detected in the patient's oral samples both pre- and post-transplant. Prior to transplantation, HHV-8 was present in the patient's WMS (WMS^s and WMS^c) and BE. Throughout the 38 wk of post-transplant sample collection, HHV-8 was present in all WMS (WMS^s and WMS^c) samples studied and in the BE for the first 4 wk. However, HHV-8 was only detected in the PS and PE at 2 wk and 4 wk post-transplant, respectively. Sixteen oral samples (16/35; 46%) were concordantly positive for KS330 and K1/V1 (WMS^s: 5; WMS^c: 6; PS: 1; BE: 3; PE: 1), 2 samples (2/35; 6%) were positive for K1/V1 only (WMS^s: 1; WMS^c: 1), and another 2 (2/35; 6%) for KS330 alone (WMS^s: 1; BE: 1) (Table 6.1). Sixteen oral samples (16/20; 80%) were also positive for long K1 DNA, 14 of which were concordantly positive for KS330 and K1/V1 (Table 6.2).

Table 6.1 HHV-8 DNA PCR findings, ELISA and viral load assessments in oral and blood samples

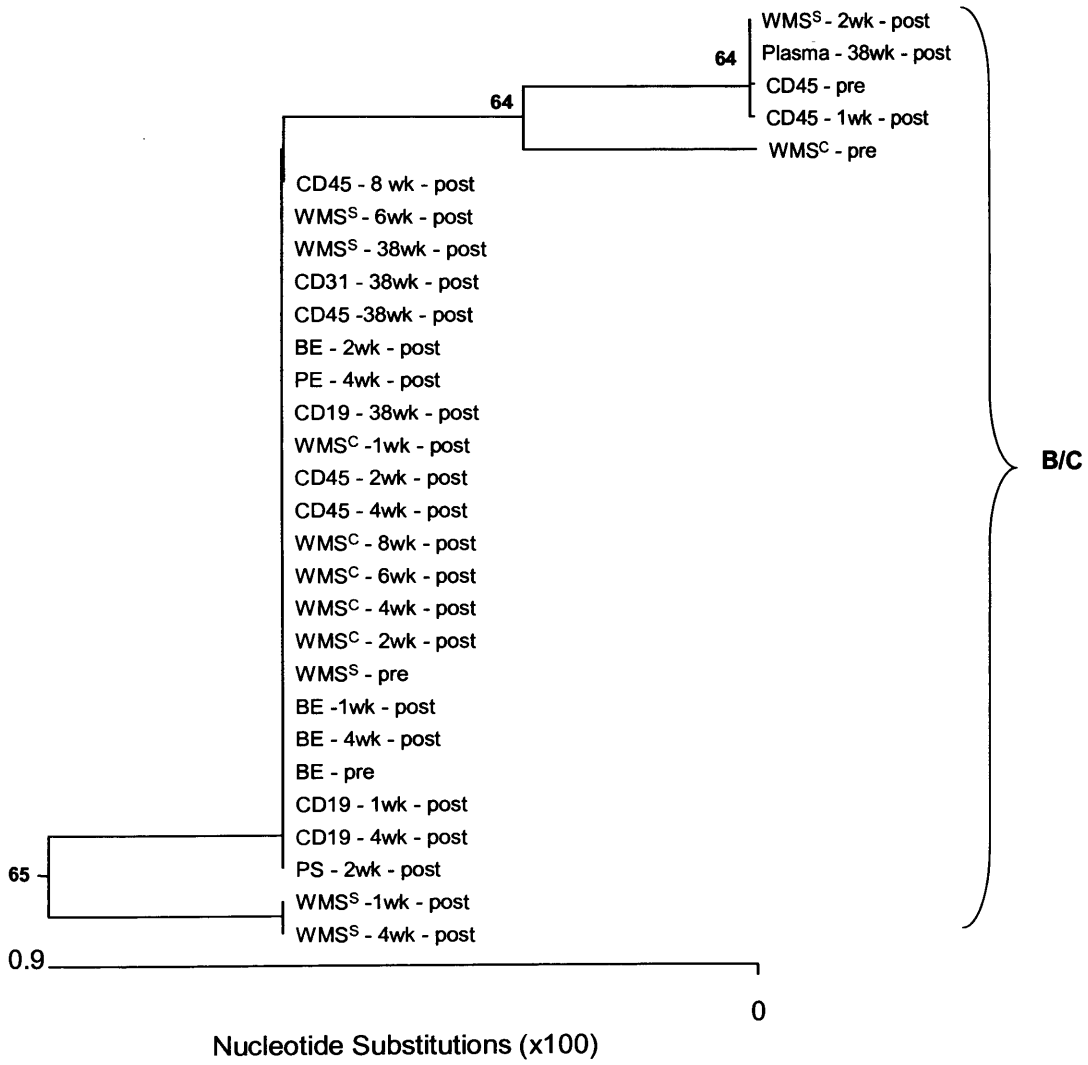
Timing	HHV-8 PCR RESULTS																						HHV-8 viral load WMS / PS (copies / ml)	ELISA
	Blood												ORAL											
	CD2		CD14		CD19		CD31		CD45		Plasma		WMS[s]		WMS[c]		PS		BE		PE			
	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330	K1/V1	KS330		
Pre	-	-	-	-	+	-	-	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	2,606,250 / NT	+
1 wk post	-	-	-	-	-	+	-	-	+	+	-	-	+	+	+	+	-	-	-	+	-	-	2,270,000 / NT	+
2 wk post	-	-	-	-	-	-	+	-	+	+	-	-	+	+	+	+	+	+	+	+	-	-	788,750 / 479	+
4 wk post	-	-	-	-	+	+	-	-	-	+	-	-	+	+	+	+	-	-	+	+	+	+	4,106,500 / NT	+
6 wk post	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	170 / NT	+
8 wk post	+	-	-	-	-	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	- / NT	+
38 wk post	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	1,495/ NT	+

WMS[s], whole mouth saliva supernate fraction; WMS[c], whole mouth saliva cellular fraction; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; +, HHV-8 DNA amplified; -, HHV-8 DNA not amplified; NT, not tested

6.3.2 KS330 nucleotide sequence diversity

A dendrogram displaying the diversity of consensus KS330 sequences and their genotypic assignments is shown in Fig 6.1. A narrow inter-sample KS330 sequence diversity ($\leq 1.2\%$) was observed. The genotype of HHV-8 in all blood and oral samples taken at different intervals was identical (genotype B/C). KS330 sequences have been deposited in EMBL Nucleotide Sequence Database (accession numbers AM745354 to AM745382).

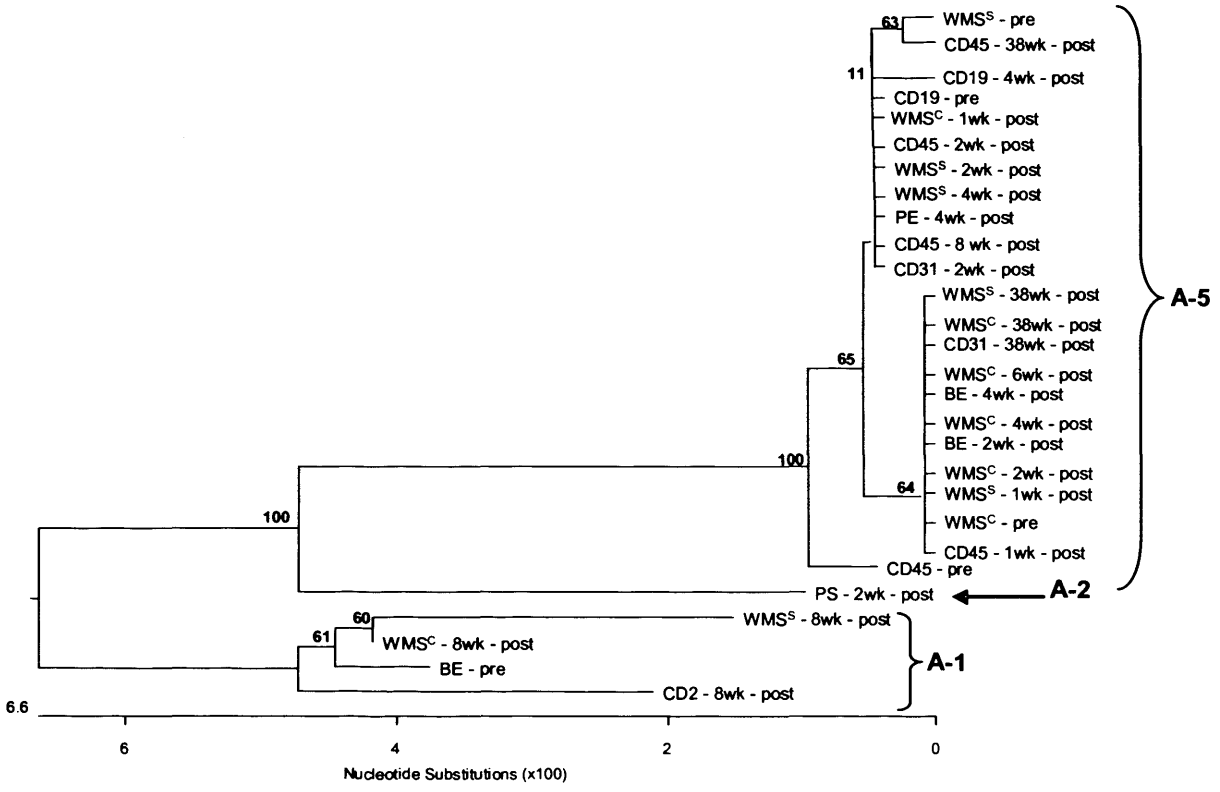
Figure 6.1 Dendrogram showing diversity of CRF-tr 4's consensus KS330 sequences. Bootstrapping for 1000 replicates is noted as a percentage at major branch points.



6.3.3 K1/V1 nucleotide sequence diversity

A dendrogram displaying the diversity of consensus K1/V1 sequences and their genotypic assignments is shown in Fig 6.2. Inter-sample sequence diversities ranged between 0% and 11.7%. Various blood and oral samples, taken pre-transplant, 2 wk and 8 wk post-transplant, segregated into two genotypes (A1 and A5 or A2 and A5), while those taken 1 wk, 4 wk, 6 wk and 9 months post-transplant all belonged to the A5 genotype (Table 6.2). K1/V1 sequences have been deposited in EMBL Nucleotide Sequence Database (accession numbers AM745383 to AM745410).

Figure 6.2 Dendrogram showing diversity of CRF-tr 4's consensus K1/V1 sequences. Bootstrapping for 1000 replicates is noted as a percentage at major branch points.

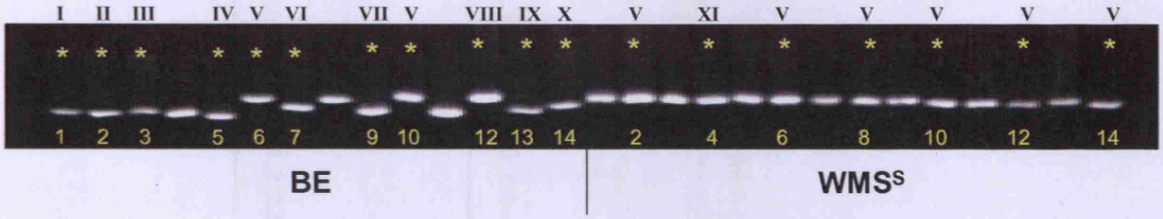


6.3.4 Inter- and intra-sample K1/V1 nucleotide sequence differences studied by the combined DGGE screening-nucleotide sequencing protocol

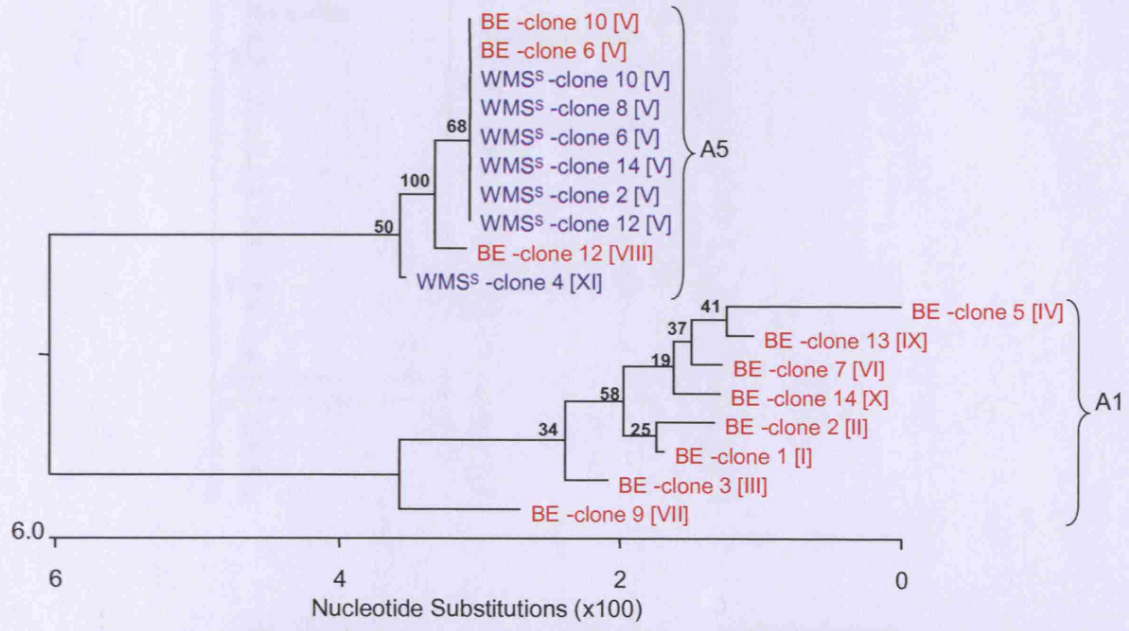
The patient's pre-transplant WMS^s, showing concordantly positive results for KS330, K1/V1 & long K1 DNA, showed a 9.5% K1/V1 consensus sequence divergence from the pre-transplant BE sample (Figure 6.2). By cloning, DGGE (Figure 6.3 (A)) and sequencing 14 colonies from the pre-transplant WMS^s and BE K1/V1 amplificates a maximum divergence of 0.5% among the WMS^s and 9.5% among the BE clonal sequences was observed. The majority sequence (V) was identical to the consensus ORF K1/V1 sequence from the pre-transplant WMS^s. The BE sequences could be assigned to belong to 2 genotypes (A1 and A5), while the WMS^s sequences all belonged to A5. In addition, a sequence diversity of up to 9.5% between the pre-transplant WMS^s clones and the majority of buccal clones was observed. A dendrogram displaying the diversity of sequences generated after sequencing a selected number of clones, indicated by (*) in Figure 6.3(A), is displayed in Figure 6.3(B). Sequences derived from the PCR clones indicated by (*) have been deposited in EMBL Nucleotide Sequence Database (accession numbers AM745411 to AM745428). A comparison of the nucleotide sequences is shown in Figure 6.3(C).

Figure 6.3 A) DGGE photograph accommodating K1/V1 DNA amplified from 14 clones generated from CRF-tr 4's pre-transplant WMS^s and BE samples. Arabic numerals represent assigned clone numbers, coinciding with lane positions in the gel; colony products with unique sequences are assigned different Roman numerals. B) Dendrogram showing phylogenetic distribution of K1/V1 consensus derived from (*) indicated clones of pre-transplant WMS^s and BE samples; Roman numerals in parenthesis denote unique sequences found within specimen; Bootstrapping for 1000 replicates is noted as a percentage at major branch points. C) A comparison of nucleotide sequences; Roman numerals denote unique sequences and numbers in the parenthesis denote the number of clones with the unique sequences. Dots indicate residues occupying positions aligned to those of the majority sequence (V) at top.

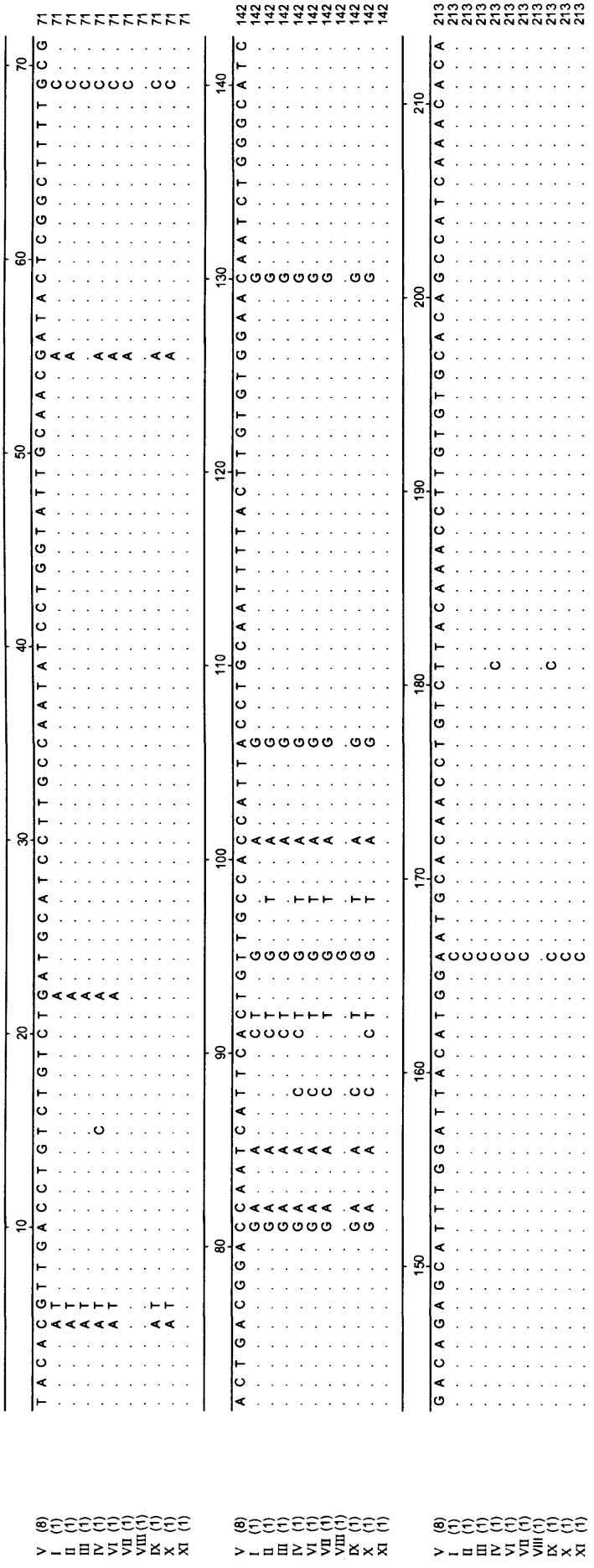
(A)



(B)



(C)



6.3.5 Long K1 nucleotide sequence diversity

The sequences of long K1 consensus all belonged to A5 genotype, with very narrow sequence diversities (≤0.1%) (Figure 6.4; Table 6.2).

Figure 6.4 Dendrogram showing diversity of CRF-tr 4's consensus long K1 sequences. Bootstrapping for 1000 replicates is noted as a percentage at major branch points.

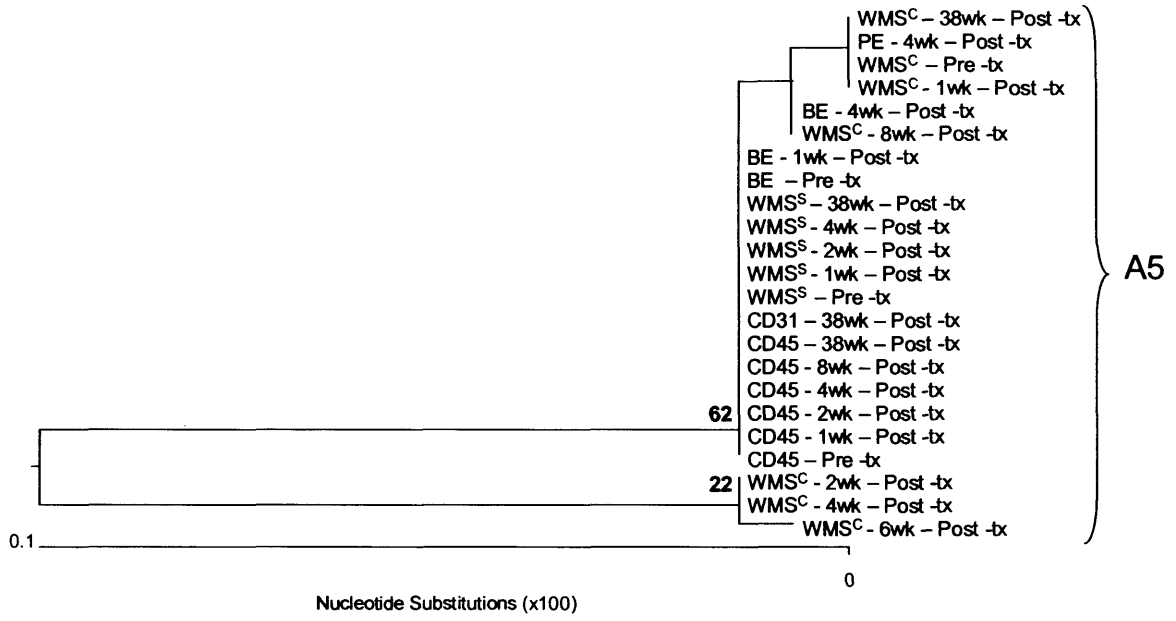


Table 6.2 Linkage analysis of KS330, K1/V1 and long K1 genotypes as determined by consensus sequencing

Timing of sample	Patient sample	KS330 genotype	K1/V1 genotype	long K1 genotype
Pre-transplant	CD19	-	A5	-
	CD45	B/C	A5	A5
	WMS^s	B/C	A5	A5
	WMS^c	B/C	A5	A5
	BE	B/C	A1	A5
1 wk post-transplant	CD19	B/C	-	-
	CD45	B/C	A5	A5
	WMS^s	B/C	A5	A5
	WMS^c	B/C	A5	A5
	BE	B/C	-	A5
2 wk post-transplant	CD31	-	A5	-
	CD45	B/C	A5	A5
	WMS^s	B/C	A5	A5
	WMS^c	B/C	A5	A5
	PS	B/C	A2	-
	BE	B/C	A5	-
4 wk post-transplant	CD19	B/C	A5	-
	CD45	B/C	-	A5
	WMS^s	B/C	A5	A5
	WMS^c	B/C	A5	A5
	BE	B/C	A5	A5
	PE	B/C	A5	A5
6 wk post-transplant	WMS^s	B/C	-	-
	WMS^c	B/C	A5	A5
8 wk post-transplant	CD2	-	A1	-
	CD45	B/C	A5	A5
	WMS^s	-	A1	-
	WMS^c	B/C	A1	A5
38 wk post-transplant	CD19	B/C	-	-
	CD31	B/C	A5	A5
	CD45	B/C	A5	A5
	Plasma	B/C	-	-
	WMS^s	B/C	A5	A5
	WMS^c	-	A5	A5

WMS^s, supernate fraction of whole-mouth saliva; WMS^c, cellular fraction of whole-mouth saliva; PS, parotid saliva; BE, buccal exfoliate; PE, palatal exfoliate; -, not detected

6.3.6 Whole mouth saliva supernate viral load

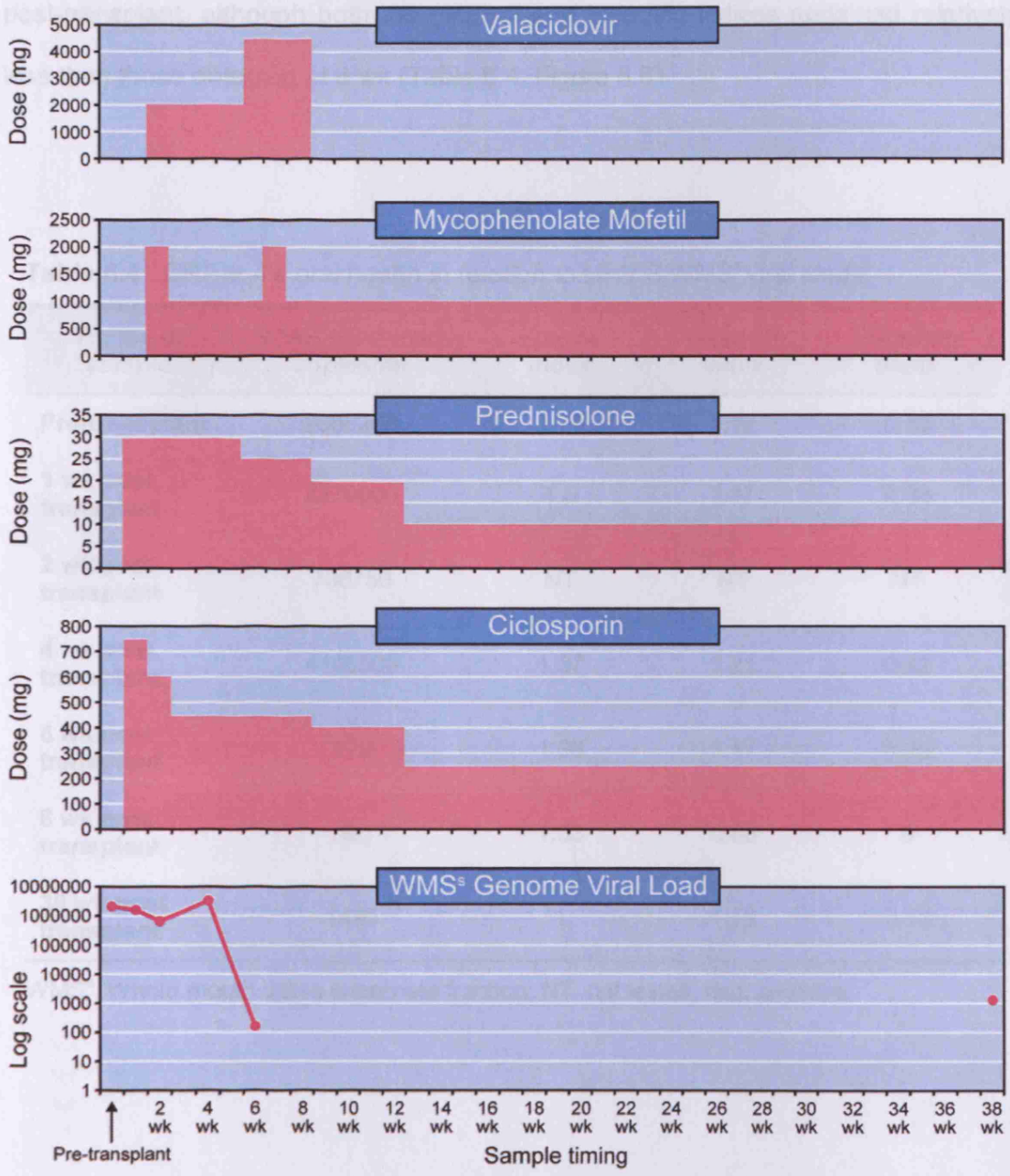
Prior to transplantation, the patient's whole mouth saliva carried 2,606,250 copies/ml. After one month of post-transplant immunosuppression, the HHV-8 WMS^s load increased to 4,106,500 copies/ml, thereafter dropping significantly, coinciding with an increase to the dosage of valaciclovir given (Table 6.3; Figure 6.5).

Table 6.3 Daily dosage of administered drugs in relation to HHV-8 WMS^s viral loads

	Viral load copies/ml	**Drug dose (mg)**			
Timing of sample	WMS^s	Ciclosporin	Prednisolone	Mycophenolate mofetil	Valaciclovir
Pre-transplant	2,606,250	0	0	0	0
1 wk post-transplant	2,270,000	750	30	0	0
2 wk post-transplant	788,750	600	30	2000	2000
4 wk post-transplant	4,106,500	450	30	1000	2000
6 wk post-transplant	170	450	25	1000	4500
8 wk post-transplant	neg	450	25	1000	4500
38 wk post-transplant	1,495	250	10	1000	0

WMS^s, supernate fraction of whole-mouth saliva; neg, negative

Figure 6.5 Time charts illustrating change in CRF-tr 4's HHV-8 WMS^s viral loads in relation to administered drug daily dosage



6.3.7 Association between oral inflammation and HHV-8 shedding

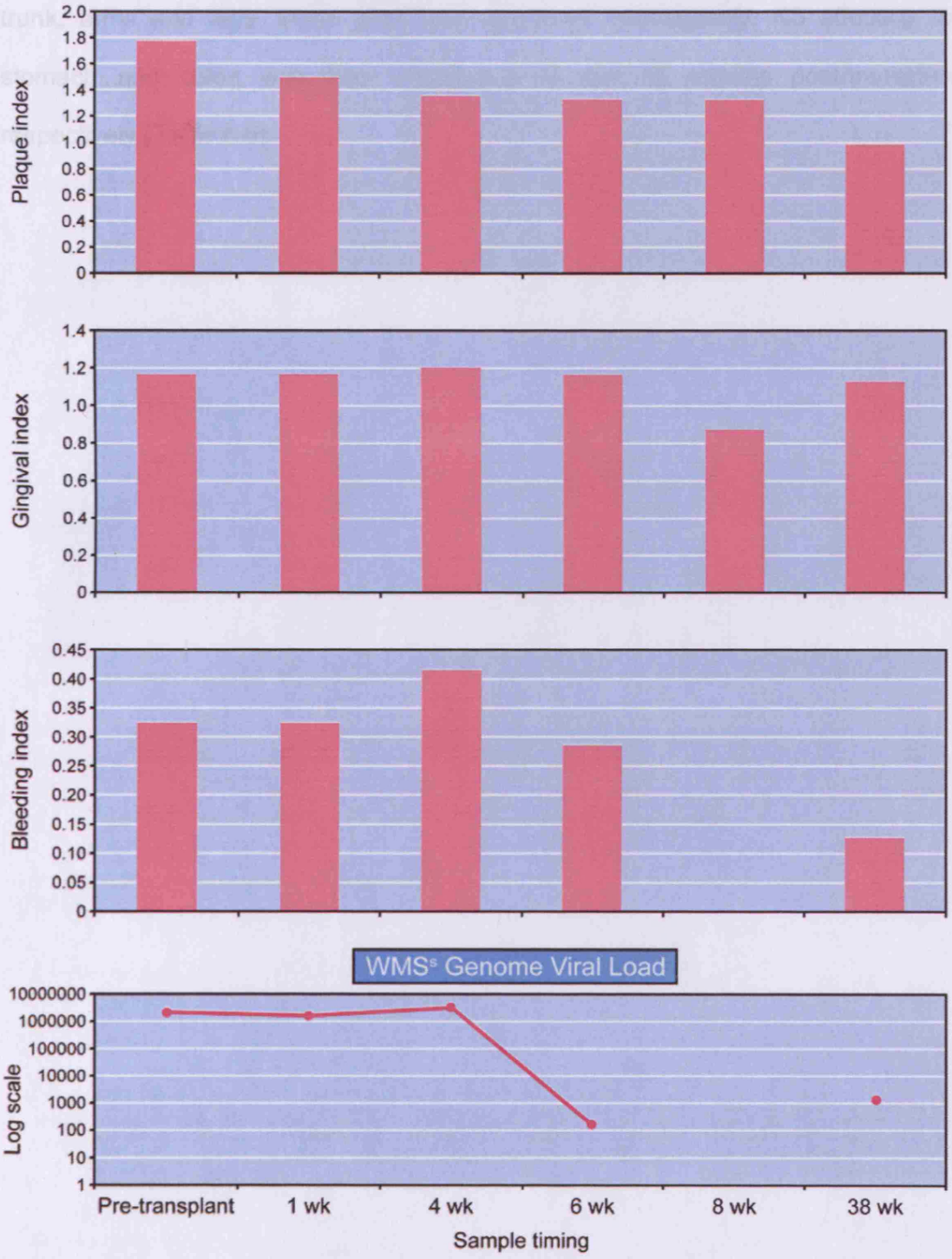
Salivary HHV-8 shedding in the first 8 wk post-transplant, as indicated by the WMS^s viral load, was shown to be associated with changes in both the gingival and bleeding oral indices. At 38 wk post-transplant, the WMS^s viral load exceeded that at 6 wk post-transplant, although both the gingival and bleeding indices remained relatively less than those detected at 6 wk (Table 6.4; Figure 6.6)

Table 6.4 CRF-tr 4's oral health in relation to HHV-8 WMS^s viral loads

Timing of sample	WMS^s viral load copies/ml	Plaque index	Gingival index	Bleeding Index
Pre-transplant	2606250	1.79	1.17	0.33
1 wk post transplant	2270000	1.5	1.17	0.33
2 wk post transplant	788750	NT	NT	NT
4 wk post transplant	4106500	1.37	1.21	0.42
6 wk post transplant	170	1.33	1.17	0.29
8 wk post transplant	neg	1.33	0.88	0
38 wk post transplant	1495	1	1.13	0.13

WMS^s, Whole mouth saliva supernate fraction; NT, not tested; neg, negative

Figure 6.6 Column and line charts illustrating CRF-tr 4's HHV-8 WMS^{s} viral loads in relation to oral indices



6.3.8 Clinical progress

The patient's clinical progress and laboratory investigations were monitored and recorded both pre- and post-transplantion, for a period of up to 34 months. Twenty-six wk following allograft receipt the patient developed cutaneous KS at the upper trunk, arms and legs, which was later confirmed histologically. KS affecting the stomach and colon was later diagnosed 14 and 15 months post-transplant, respectively (Table 6.6).

Table 6.5 Summary of CRF-tr 4's clinical progress and outcomes of laboratory investigations

Timing (Date)	Clinical presentation & investigation
Pre-transplant (2002/2003)	HCV Antibody and PCR +ve Liver biopsy → HCV liver cirrhosis; administered Interferon and Ribavirin
Pre-transplant (2003)	End stage renal disease on regular haemodialysis 3x week; controlled hypertension (5mg Amlor once daily)
(April 2004)	**Left Renal allograft transplant**
8 wk post-transplant (May 2004)	Developed post transplant Diabetes Mellitus (DM) → Insulin prescribed
26 wk post-transplant (Sept 2004)	**Developed multiple nodules over the upper trunk, arms and legs**
38 wk post-transplant (Dec 2004)	Skin punch biopsy taken from upper trunk → **Dx: KS**
10 months post-transplant (Jan 2005)	Gastroscopy & colonoscopy → multiple extensive polypoid lesions in stomach and duodenum **resembling KS** Biopsy from stomach and rectum → Dx: negative for KS
12 months post-transplant (April 2005)	No new skin lesions; upper arm lesions disappeared
14 months post-transplant (June 2005)	Gastric Biopsy → DX: **Focal area suspicious for KS** + Mild chronic gastritis
15 months post-transplant (July 2005)	Colon Biopsy → DX: **Focal area of KS** and chronic colitis
16 months post-transplant (Aug 2005)	All KS skin lesions flat or disappeared; start sirolimus (rapamune®) with an overlap period with ciclosporin, until rapamune level achieved.
17 months post-transplant (Sept 2005)	Ciclosporin stopped Allograft maintained on prednisolone + sirolimus immunosuppressive regimen

Table 6.6 Summary of patient's clinical progress and outcomes of laboratory investigations... *continued*

Timing post-transplant (Date)	Clinical presentation & investigation
19 months post-transplant (Nov 2005)	Patient complained of abdominal pain → abdominal ultrasound and liver biopsy
20 months post-transplant (Dec 2005)	Skin KS completely disappeared Liver biopsy result → no fibrosis, very mild portal inflammation & lobulitis. Improved liver function with reduction of Rapamune dose
28 months post-transplant (Aug 2006)	Liver Ultrasound → fatty liver infiltrate Thyroid Ultrasound → bilateral thyroid nodules
31 months post-transplant (Nov 2006)	Oedema and thick skin of lower limbs → starting 40 mg furosemide (Lasix®) once daily. Bilateral thyroid nodules → referral to Endocrinologist Abnormal liver function test → referral to Hepatologist
34 months post-transplant (Feb 2007)	Transplanted kidney → stable Lower limbs → Oedema, mild pitting and thick skin → starting 25mg Atenolol once daily

6.4 Discussion

Although KS was diagnosed by histopathological examination of KS tissues on week 38 post-transplant, analysis showed that HHV-8 DNA was present in a variable number of successive pre-KS oral and blood samples. Among the various pre-KS tests conducted, saliva HHV-8 PCR and plasma anti-HHV-8 testing proved to be consistently positive. WMS yielded higher PCR amplification rates than other oral samples, likely reflecting HHV-8 shed from other sites, such as tonsils (Koelle *et al.*, 1997; Chagas *et al.*, 2006b) or tongue (Triantos *et al.*, 2004; Widmer *et al.*, 2006). Given that the WMS^s HHV-8 viral load was found to be directly related to the gingival bleeding index, blood present in the crevicular fluid could have contributed to HHV-8 in saliva as well. Such association between inflammation in the oral cavity and HHV-8 salivary viral load was previously reported elsewhere using leukocyte esterase chemical indicator strips to assess oral inflammation semi-quantitatively (Casper *et al.*, 2004) rather than oral gingival indices. The association observed in this case between inflammation in the oral cavity and HHV-8 WMS^s viral load, as indicated by both the gingival and bleeding oral indices, may be coincidental, however, especially as it could not be verified when a larger cohort of Saudi Arabian end-stage renal disease patients and allograft recipients was examined (Chapter 3). In addition, while HHV-8 has been detected in the periodontal tissues of HIV infected individuals without KS, it has not been observed in periodontitis sites of non-HIV-infected persons (Mardirossian *et al.*, 2000; Contreras *et al.*, 2001).

Salivary HHV-8 shedding in immunocompromised individals may be very high, even before immunosuppression is administered, and increase further when immunosuppression is started. The salivary HHV-8 genome viral load, in this case, was found to increase by 60% following one month of immunosuppression, thereafter dropping significantly, coinciding with an increase to the dosage of valaciclovir given.

Valaciclovir is an ester of aciclovir (antiviral drug), which is licensed for herpes zoster and herpes simplex infections of the skin and mucous membranes. Aciclovir has previously been found to have some, although minimal, effects on the inhibition of HHV-8 DNA production (Neyts and De Clercq, 1997; Kedes and Ganem, 1997; Medveczky *et al.*, 1997). This relatively weak effect may explain why, in this case, higher doses of valaciclovir were required to produce a significant reduction in the WMS^s HHV-8 viral load. Noteworthy is the observation that the HHV-8 load in WMS^s later increased following the cessation of valaciclovir, substantiating the effect of aciclovir. Nevertheless, valaciclovir administered throughout wk 2 to 8 post-transplant, as a means of prophylaxis against herpes infection, was inefficient in the prevention of KS. This outcome is in agreement with previous studies showing aciclovir having no affect on the development of KS in AIDS patients (Mocroft *et al.*, 1996; Glesby *et al.*, 1996), although it can be argued that the early cessation of valaciclovir could have attributed to the development of KS in this case.

Previous studies have detected HHV-8 DNA in the buccal mucosa and palate of KS patients (Cook *et al.*, 2002a; Cook *et al.*, 2002b; Beyari *et al.*, 2003) and in the normal oral mucosa of healthy immunocompetant individuals (Duus *et al.*, 2004). The present study supports these findings, but in the context of an iatrogenically suppressed individual. HHV-8 DNA was found in both pre- and post-transplant BE when WMS^s viral loads were $\geq$788750 copies/ml and in post-transplant PE when the WMS^s viral load was at its highest, indicating that HHV-8 in the buccal and palatal cells contributed to the increased WMS^s HHV-8 viral load or vice versa. The inability to detect HHV-8 DNA in the BE or PE at other intervals does not rule out the possibility that the virus may still replicate in areas of the palatal or buccal mucosa further away from the selected sampling site. The limited HHV-8 DNA detection rate in saliva collected from the parotid duct supports previous findings indicating that major salivary glands are not important contributors to HHV-8 shedding (Pauk *et al.*,

2000).

To assure broad representation of the blood subpopulations, a variety of which have previously been reported to support HHV-8 persistence (Table 1.4), immunomagnetic fractioning of CD45+, CD31+, CD19+, CD14+ and CD2+ cell subsets was conducted and all the fractions tested for HHV-8 infection. HHV-8 DNA was detected in the PBMCs before the development of clinically evident KS and even before renal transplant, contrary to previous reports of HHV-8 genome being present in mainly PBMCs of people with active KS (Purvis *et al.*, 1997; Metaxa-Mariatou *et al.*, 2004). The detection rate of HHV-8 in PBMCs varied according to the cell subsets studied, with the CD45+ cells generating the most positive results (6/7), followed (in descending order) by: CD19+ cells (4/7), CD31+ cells (2/7) and CD2+ cells (1/7). The CD14+ cells, previously reported to harbour HHV-8 in patients with KS (Henry *et al.*, 1999; Pellet *et al.*, 2006), were negative for HHV-8 DNA throughout the entire sampling period. This result may reflect a specific HHV-8 cellular tropism or may be a result of a low copy number-carriage of viral DNA that was below the PCR detection limit. Likewise, the failure to detect HHV-8 at 6 wk post-transplant, in any of the blood cell subsets, may be due to a low viral load (Decker *et al.*, 1996; Blackbourn *et al.*, 1997), possibly due to the increase in dosage of valaciclovir, or intermittent shedding of HHV-8 DNA by PBMCs (Laney *et al.*, 2004).

HHV-8 plasma viraemia was found only upon the development of clinical KS. This observation is in agreement with previous work where HHV-8 plasma viraemia was found to be positively associated with active disease (Harrington *et al.*, 1996; Tedeschi *et al.*, 2001; Campbell *et al.*, 2003; Duus *et al.*, 2004).

The HHV-8 detection rate in oral and blood samples varied according to the amplified HHV-8 genome region, with 23/77 (30%) of samples being concordantly positive for

KS330 and K1/V1, 6/77 (8%) positive for KS330 alone and 5/77 (7%) positive for K1/V1 alone. HHV-8 in WMS was amplified from a single region only when WMS^s viral load was low ($\leq$170 genome-equivalents/ml). Therefore, single region-positivity in other samples may also reflect a low viral load.

The extreme polymorphism of ORF K1 was again exploited to investigate the genomic diversity of HHV-8 in different anatomical compartments of the patient through amplification of K1/V1 and long K1 segments from ORF K1. The K1/V1 sequencing led to the assignment of sequences originating from the patient's pre-transplant BE, 8 wk CD2, WMS^s and WMS^c samples to the A1 genotype, from the patient's 2 wk PS to the A2 genotype, and the remaining samples to the A5 genotype (Fig 6.2). These findings point to inter-compartmental (blood vs. oral) and intra-compartmental (oral) carriage of multiple HHV-8 strains. The long K1 segment sequencing, on the other hand, led to the assignment of all sequences to the A5 genotype, including the pre-transplant BE and 8 wk WMS^c previously assigned by K1/V1 sequencing to A1 genotype.

Long K1 could not be amplified from the remaining K1/V1 genotype A1 samples (8 wk CD2 and WMS^s). This may be explained by the fact that long K1 segment, being >3 times longer than K1/V1, confers lesser sensitivity to PCR detection. The absence of long K1 amplification could also be due to the presence of a new unidentified hybrid HHV-8 strain carrying a K1/V1 A1 genotype, with a primer binding site sequence preventing the annealing of the long K1 primers used. It has recently been reported that the HHV-8 K1 C7 genotype shows a difference in the nucleotide sequence at the long K1 inner forward primer binding site (Duprez *et al.*, 2006). Additionally, the genotyping of HHV-8 strains have been continuously modified with the description of new strains from different geographical areas (Kajumbula *et al.*, 2006; Hayward and Zong, 2007); thus other strains may yet be identified (Duprez *et*

al., 2006).

The dominant A5 variant, reported here, has previously been identified in African (Zong *et al.*, 2002; Treurnicht *et al.*, 2002; Mbulaiteye *et al.*, 2006; Hayward and Zong, 2007) and Brazilian (Nascimento *et al.*, 2005) samples, but not in Saudi samples. It has been suggested that the A5 variant may be more efficiently transmitted as it has been detected more frequently in children than in mothers (Mbulaiteye *et al.*, 2006).

Direct sequencing studies have an important limitation in that they are unable to produce consensus sequence data that will allow sequences of minority variants to be identified. To overcome this limitation, the combined DGGE screening-nucleotide sequencing protocol was applied to K1/V1 amplicons generated from clonal inserts. To reduce the degree to which PCR generates nucleotide misincorporation, high-fidelity PCR was applied to sample extracts. Applying this combined DGGE-sequencing protocol showed that the patient's pre-transplant BE clones exhibited a significant sequence diversity of up to 9.5%, segregating BE clones into two genotypes (A1 and A5), with the minority clade (A5) comprising sequences genotypically distinct from the majority clade (A1). The findings from this patient are supported by other reports (Gao *et al.*, 1999; Beyari *et al.*, 2003) but contrast with some studies in individuals with (Meng *et al.*, 2001; Stebbing *et al.*, 2001; Zong *et al.*, 2002) and without (Mbulaiteye *et al.*, 2006) KS, which have not revealed evidence for intra-person variation in HHV-8 sub-genomic sequences. Unfortunately, KS biopsy samples for this patient could not be retrieved, so the origin of the viral strain that led to KS in this patient could not be defined.

Because HHV-8 genomic sequences carried in this patient's blood and oral samples could belong to either genotype, conclusions cannot be drawn about the selective

compartmental tropism of HHV-8 strains. Current evidence from other studies also suggests that HHV-8 exhibits broad tropism (Dupin *et al.*, 1999; Pauk *et al.*, 2000; Blackbourn *et al.*, 2000).

Upon continuous thorough oral examination, no oral KS, specifically palatal KS, could be detected at any time. The time between kidney transplant and KS presentation ($\approx$ 6 months) or actual diagnosis ($\approx$ 9 months) for this patient, was less than that previously reported in Saudi Arabian renal allograft recipients (Qunibi *et al.*, 1988). The first presentation of KS was in the form of multiple nodules affecting the skin of the upper trunk, arms and legs. This was followed by the diagnosis of focal areas of KS in the stomach and colon at 14 and 15 months post-transplant respectively, consistent with previous reports of skin and gastrointestinal tract KS in Saudi Arabian renal transplant recipients (al Sulaiman and al Khader, 1994) and reports of skin KS mostly affecting the lower extremities (Moosa, 2005).

Two months after the histopathological diagnosis of abdominal KS, the patient was started on a relatively new immunosuppressant (sirolimus). A month later, when the sirolimus blood level was achieved, ciclosporin was stopped and the allograft maintained on a regimen of prednisolone and sirolimus. Conversion from calcineurin inhibitors such as ciclosporin, to sirolimus has been reported as an option for renal transplant patients who develop a tumour (Sanchez-Fructuoso *et al.*, 2006; Lebbe *et al.*, 2006; Campistol and Schena, 2007) and can result in the regression of KS (Campistol and Schena, 2007; Volkow *et al.*, 2007; Yilmaz *et al.*, 2007; Hassan *et al.*, 2007) and may prevent its recurrence (Segoloni *et al.*, 2007). Sirolimus exhibits antiangiogenic activity related to impaired production of vascular endothelial growth factor (VEGF) and limiting proliferative response of endothelial cells to stimulation by VEGF, limiting the progression of KS (Guba *et al.*, 2002). The skin KS lesions in this case completely disappeared 11 months after their initial clinical appearance and 3

months after the cessation of ciclosporin.

This case study involving a prospectively-studied individual undergoing renal allograft transplant again identifies the oral cavity as a major site of HHV-8 shedding, suggesting that asymptomatic and persistent oral HHV-8 viraemia, before or after renal allograft transplantation may precede the appearance of iatrogenic-KS lesions and may also predict the development of KS. Furthermore, such patients may be infected by multiple viral strains, implying their poor immunity from past infection. Hence, monitoring potential transplant recipients for HHV-8 oral infection may be useful for developing therapeutic and prophylactic strategies using antiviral agents, known to inhibit HHV-8 production (e.g., ganciclovir, foscarnet and cidofovir).

Chapter 7

Summary, Conclusions and Suggestions for Further Work

7.1 Summary and significance of results

Transplantation-associated KS is more prevalent among renal allograft recipients in Saudi Arabia than many other geographic regions of the world. The present study has been undertaken to evaluate the extent of oral and blood HHV-8 shedding and the genomic diversity of the virus involving samples collected from eight study groups representing Saudi Arabian populations with different risks of HHV-8 infection. This study further explores the factors that may influence the shedding of the virus in the groups in which the prevalence of HHV-8 infection is particularly high. The study was primarily cross sectional in design, with a prospective component involving chronic renal failure patients undergoing kidney transplant.

The findings reported in this thesis substantiate the hypothesis that a disparity in HHV-8 prevalence rates exists between the Saudi Arabian general population and patients with kidney disease. A higher HHV-8 seroprevalence was observed in Saudi Arabian patients with renal disease than in the general population. Plasma HHV-8 IgG was detected in all renal allograft recipients with a history of KS (the RAR-KS group) and in all pre-KS samples from a CRF patient undergoing renal transplant (CRF-tr 4), thus showing high degree of concordance between HHV-8 seropositivity and KS.

When evaluating the sub-cellular distribution of HHV-8 DNA detection, HHV-8 DNA was detected in CD2+, CD19+, CD31+ and CD45+ cells with some variation in the rate of detection, suggesting that HHV-8 is pleotropic for a wide variety of cells circulating in the blood. In the case of CRF-tr 4, who later developed KS, the amplification rate of HHV-8 sub-genomic DNA by nested PCR in CD45+ cells compared to the rates in other cell populations was consistently higher. This finding suggests that the principal cell types in peripheral blood carrying HHV-8 are not B cells, T cells, monocytes or endothelial cells.

HHV-8 DNA was found to be more frequently detectable in the oral samples of CRF patients than in blood, reflecting the high load of shedding into the oral cavity and possible lower replicative activity of the virus systemically. Among the various pre-KS PCR tests conducted on CRF-tr 4, saliva HHV-8 PCR testing consistently proved to yield positive results. Additionally, the viral load in the cell-free fraction of whole-mouth saliva, throughout this study, ranged between approximately 1.7×10^2 and 1.2×10^8 genome-copies/ml. Thus, in the patients studied, salivary shedding of HHV-8 was frequent and could be extensive. Moreover, the number of renal disease patients with detectable HHV-8 DNA in their whole saliva samples exceeded those with detectable HHV-8 DNA in their buccal or palatal exfoliates. This lower HHV-8 DNA detection rate in buccal and palatal exfoliates compared to whole saliva probably reflects active HHV-8 replication in the oral epithelium other than the buccal mucosa or hard palate or in areas of the palate or buccal mucosa further away from the selected sampling sites. Thus, patients with both buccal and palatal exfoliates that were concordantly positive for K1/V1 and KS330 HHV-8 DNA showed the highest WMS^s viral load DNA when compared to others in the group with a detectable WMS^s viral load (patient CRF 24: 119,562,500 genome-copies/ml; RAR-KS 2: 2,198,250 genome-copies/ml; and CRF-tr 4: 4,106,500 genome-copies/ml at week 4 post-transplant). These findings indicate that HHV-8 in their buccal and palatal cells contributed to the increased WMS^s HHV-8 viral load.

Results from the present study suggest that HHV-8 carriage in saliva is independent of its carriage in blood. Further, the virus in saliva may not be due to transfer from blood to the mouth, since HHV-8 was not always present simultaneously in both blood and saliva and no association between inflammation in the oral cavity and HHV-8 shedding was observed in all but one patient (CRF-tr 4).

The current study is the first to report the identification of A2, A3, A5 and B HHV-8 K1

genotypes in the Saudi Arabian population. When a 840-bp fragment of HHV-8 ORF K1 (long K1), which encompasses both HHV-8 ORF K1 VR1 and VR2, was amplified, sharper discrimination between A and C genotypes was possible. However, long K1 could not be amplified from some K1/V1 genotype A1 samples. This may be explained by the fact that long K1, being >3 times longer than K1/V1, confers lesser sensitivity to PCR detection. The absence of long K1 amplification may also be due to the presence of a new unidentified hybrid HHV-8 strain. Because overall HHV-8 genomic sequences carried in blood and oral samples could belong to either genotype, conclusions could not be drawn about the selective tropism of HHV-8 strains.

Whether intrahost HHV-8 subgenomic sequence polymorphism exists among and within oral and blood samples of renal disease patients was also investigated. Sequence variation was sought in DNA segments derived from HHV-8 ORFs 26 and K1 using both direct sequencing and PCR combined with DGGE analysis of K1/V1 clones. Intraperson genotype and subgenotype K1/V1 sequence differences were found among samples from chronic renal failure patients and renal allograft recipients. Such multiple HHV-8 carriage may reflect simultaneous coinfection by more than 1 HHV-8 strain, reactivation of latent strains, or superinfection. If it reflects superinfection, then such transmissions may not be prevented by vaccination. Nonetheless, although the immune system might not prevent superinfecting virus from initiating replication in the host, it might, after vaccination, prevent the further spread of the initial infecting strain (following reactivation) and of subsequent superinfecting strains.

As an extension of the study of polymorphism in HHV-8 subgenomic sequences ampilified from blood and oral samples, KS biopsy specimens of five patients with a history of KS were retrieved and tested. The extent of HHV-8 biopsy sequences

variation derived particularly from K1 was examined and compared with sequences derived from oral samples of the same individual. Amongst the five patients, genotypically distinct viral strains were evident: intra-lesionally in 1 patient; intra-orally in 1 patient; between an oral sample and biopsy in 2 patients; and in 4 patients, between an oral sample and plasma, and between plasma and biopsy. While the findings in these patients again substantiate multiple HHV-8 infection, the cross-sectional sampling prevented the identification of which body compartment harboured HHV-8 strains that became associated with KS development.

In the present study, there was a marked disparity in HHV-8 DNA detection rates according to the criteria used to consider HHV-8 DNA as being detectable: PCR positivity for KS330 alone, for K1/V1 alone, or for both KS330 and K1/V1. Great care was taken to rule out possible cross contamination as contributing to solitary K1/V1 or KS330 reactivities. Samples found with identical K1 sequences and those with single K1 or KS330 positivities underwent another round of DNA extraction and amplification. Furthermore, nucleotide sequencing of the positive control (HHV-8 infected BCBL-1 cell line) was carried out for comparison, and its KS330 and K1/V1 sequences were determined to be different from the KS330 or K1/V1 sequences of patient samples. Previous studies into HHV-8 genome detection rates in KS patients from Malawi (Beyari *et al.*, 2003) and blood donors from the UK (Kumar *et al.*, 2007) using identical PCR conditions for the amplification of both K1/V1 and KS330 detected similar discordance between K1 and KS330 in a small number of their samples, especially from people who were anti-HIV seronegative (Beyari *et al.*, 2003). This discordant result may be due to a low copy number-carriage in them of HHV-8 DNA. In the present study, HHV-8 in WMS^s was amplified from a single region only when WMS^s viral load was low ($\leq$170 genome-equivalents/ml).

The present study is limited by several factors. The first is the cross-sectional nature

(in most part), which precludes resolution of whether concurrent HHV-8 infections in the patients resulted from co-infection or super-infection and identification of episodic HHV-8 shedding. Second, buccal and palatal sample collection from renal disease patients was restricted to a single sample for each investigated area from each patient, which may not be representative of the entire anatomical area. Third, saliva and oral cells samples were not collected from the healthy control groups to allow comparison with renal disease patients with regard to HHV-8 oral shedding. Additionally, only CD45+ cell subsets were isolated from the control group's whole blood samples, preventing a comprehensive comparison between blood HHV-8 genoprevalence amongst the study groups.

7.2 Conclusion

Several explanations can be given for the approximately 10-fold increased incidence of KS in Saudi Arabian renal transplant recipients by comparison to such patients in other areas. One is that the background prevalence of HHV-8 infection is unusually high in the Saudi Arabian population. Findings from the present study and previous studies (Qunibi *et al.*, 1998; Almuneef *et al.*, 2001; Alzahrani *et al.*, 2005) suggest that this is not the case. A second possibility is that HHV-8 infection in Saudi Arabia occurs more frequently via allografts from infected donors. This hypothesis was tested directly in the present study which revealed that HHV-8 transmission via organ transplantation is unlikely. A third possibility is that immunosuppressive regimens used in the post-transplant management are substantially different from those used in other countries, but the present study found no indication that this was so, and, further, no association was observed in the present study between type, dose or duration of immunosuppressives and HHV-8 infection. The last possibility, and the one favoured here, is that HHV-8 transmission occurred iatrogenically in patients with chronic renal disease in the course of their receiving health care, particularly during haemodialysis when the patients are accommodated in proximity to each other, and

there is close contact with or transfer of saliva of infected individuals. Upon renal allograft receipt, these patients are further subjected to immunosuppressives (ciclosporin, prednisolone, mycophenolate mofetil, etc.), some of which have been known to have a cancer-promoting effect, which further predispose already infected transplant recipients to develop KS.

The results of this study support the recommendation to adopt strict measures to control nosocomial, salivary spread of HHV-8 to pre- and post-transplantation patients, particularly in health-care facilities that accommodate them at close quarters without regard to their susceptibility, immunity and infectivity status. Guidelines for infection control in such facilities would need to stipulate that immunocompromised patients minimise contact with human saliva. For control and prevention of post transplantation KS specifically, these measures would complement others that are being evaluated to reduce its incidence, e.g., antibody screening of organ donors and recipients, and those to effect its regression after disease has developed, e.g., conversion to the use of sirolimus as immunosuppressant. Moreover, monitoring potential transplant recipients for HHV-8 oral infection could be useful for developing therapeutic and prophylactic strategies against post-transplantation KS.

7.3 Suggestions for further studies

- Further work to clarify the oral HHV-8 genoprevalence in Saudi Arabian healthy individuals should be conducted. As HHV-8 shedding in saliva may be episodic in some patients and missed when only single specimens are analyzed (Koelle *et al.*, 1997; Pauk *et al.*, 2000), it would be necessary to serially sample from the oral cavity.

- Further larger-scale studies comparing groups on different immunosuppressive regimens and including other factors, such as the length of exposure to a specific immunosuppressive drug, may be required to study possible associations between

immunosuppressive drugs and HHV-8 infection, viral load and KS.

- In order to accurately assess the differences in CRF patients HHV-8 sero- or geno-prevalence rates according to the different areas of the country, it is proposed that a multi-centre study be conducted involving haemodialysis units from a wider sample of Saudi Arabian administrative areas. Moreover, evaluating the prevalence of HHV-8 infection in renal dialysis unit health care workers may be beneficial.

- Prospective studies evaluating the prevalence of HHV-8 in ESRD patients upon initiation and at different stages of haemodialysis are needed to determine the risk of acquiring HHV-8 infection in the dialysis unit.

- There is a need to proceed with larger-scale prospective studies evaluating the prevalence of HHV-8 in organ recipients and corresponding donors, including cadaver donors, to determine the risk of post-transplantation KS, its relationship to HHV-8 reactivation and organ-related transmission, and the morbidity and mortality due to KS.

- The design of the current study was confined to examination of HHV-8 carried in the blood, mouth and KS biopsy samples. Sampling of other body sites might reveal yet more evidence of multiple HHV-8 carriage. It may be useful to examine tissue specimens from various organs, e.g. lung, brain, spleen, liver, or possibly non-oral mucosal surfaces obtained from renal allograft recipients who died from KS, in order to confirm the presence of HHV-8 sequences in these tissues.

- Further studies should be undertaken involving the selection of HHV-8-infected individuals and the use of larger blood volumes to undertake immunomagnetic cell separation for a wider array of cell sub-types to further characterise which cell type

carries HHV-8.

- Although all possible measures were undertaken to reduce the contamination of BE and PE with saliva, there remains the possibility that contamination contributes to the positive detection rates. In order to overcome this, future studies using in situ hybridisation for HHV-8 mRNA or DNA in these exfoliates may be advantageous.

- Further attempts to amplify long K1 through altering PCR conditions and usage of different sets of primers may be necessary to explore the possibility of the presence of yet unidentified HHV-8 strains.

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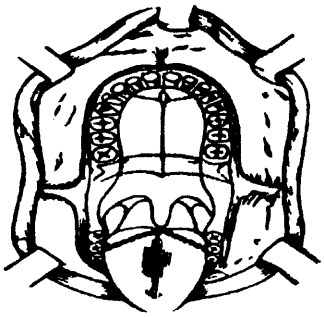
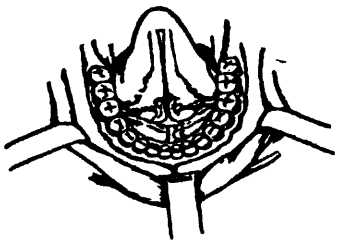
Appendices

Appendix 1. Template for intra-oral findings





Missing teeth #: _____

		#16	#12	#24	#36	#32	#44	Mean
Plaque Index	buccal							
	mesial							
	lingual							
	distal							
	Mean PI							
Gingival Index	buccal							
	mesial							
	lingual							
	distal							
	Mean GI							
Bleeding Index	buccal							
	mesial							
	lingual							
	distal							
	Mean BI							

Criteria for scoring:

- **The plaque index system (Silness J and Loe H, 1964)**

0 = No plaque in the gingival area.

1 = A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may only be recognized by running a probe across the tooth surface.

2 = Moderate accumulation of soft deposits within the gingival pocket, on the gingival margin and/ or adjacent tooth surface, which can be seen by the naked eye.

3 = Abundance of soft matter within the gingival pocket and/or on the gingival margin and adjacent tooth surface.

- **Criteria for the gingival index system (Loe H and Silness J, 1963) ***

  0 = Normal gingival

  1 = Mild inflammation – slight change in colour, slight oedema. *No bleeding on probing.*

  2 = Moderate inflammation – redness, oedema and glazing. *Bleeding on probing.*

  3 = Severe inflammation – marked redness and oedema. Ulceration. *Tendency to spontaneous bleeding.*

- **Criteria for the bleeding index system (Muhlemann and Son, 1971)**

  0 = No bleeding on blunt probing

  1 = Bleeding on blunt probing up to 30 seconds later

  2 = Immediate bleeding on probig

  3 = Spontaneous bleeding

Appendix 2. Publications and conference presentations

Publications

Al-Otaibi LM, Ngui SL, Scully CM, Porter SR, Teo CG (2007). Salivary human herpesvirus 8 shedding in renal allograft recipients with Kaposi's sarcoma. *J Med Virol.* Sep 79(9):1357-65.

Al-Otaibi LM, Al-Sulaiman MH, Porter SR, Teo CG. Oral and haematological carriage of human herpesvirus 8 prior and following onset of Kaposi's sarcoma in a renal transplant recipient. (paper submitted for publication)

Oral and poster presentations

Al-Otaibi LM, Teo CG, Porter, SR. Oral carriage and shedding of human herpesvirus 8 in a group of renal allograft recipients with a history of immunosuppression-related Kaposi's sarcoma (poster presentation). The British Society for Oral Medicine Annual Scientific Meeting, London, 11th -12th May 2006.

Al-Otaibi LM, Moles DR, Porter, SR. Oral carriage and shedding of human herpesvirus 8 in patients with renal disease (oral presentation). 8th Biennial Congress of the European Association of Oral Medicine, Zagreb, Croatia, 31st August - 2nd September 2006.

Al-Otaibi LM, Teo CG, Porter, SR. Persistent human herpesvirus 8 oral shedding and viraemia prior to Kaposi's sarcoma development in a renal allograft recipient (poster presentation). The British Society for Oral & Maxillofacial Pathology and the British Society for Oral Medicine Joint Annual Scientific Meeting, London, 23rd - 24th May 2007.

Appendix 3. Reproduction of publication reprint

Journal of Medical Virology 79:1357–1365 (2007)

REUTERS

Clinical

Normal Version

Herpesvirus infection risk high in Middle Eastern kidney transplant recipients

by David Douglas

Last Updated: 2007-09-20 16:24:12 -0400 (Reuters Health)

J Med Virol 2007;79:1357-1365.